VASCULAR DIVERSITY AND THE FUNCTIONAL ROLE OF PEPTIDASES IN ANGIOGENESIS

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List of original publications

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Abbreviations

α-SMA	α-smooth muscle actin
aFGF	acidic fibroblast growth factor
ACE	angiotensin converting enzyme
ALK	activin receptor-like kinases
Ang-1	angiopoietin-1
Ang-2	angiopoietin-2
AngI	angiotensin I
AngII	angiotensin II
AngIII	angiotensin III
AngIV	angiotensin IV
APA	aminopeptidase A
APN	aminopeptidase N
ARNT	aryl hydrocarbon receptor nuclear translocator
bFGF	basic fibroblast growth factor
BRASIL	biopanning and rapid analysis of selective interactive ligands
CAM	chick embryo chorioallantoic membrane
CEP	circulating endothelial progenitor cell
DR	diabetic retinopathy
E	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
EG-VEGF	endocrine-gland-derived vascular endothelial growth factor
FITC	fluorescein isothiocyanate
FGFR	fibroblast growth factor receptor
GH	growth hormone
IGF-1	insulin-like growth factor-1
HB-EGF	heparin binding EGF-like growth factor
HIF-1	hypoxia-inducible transcription factor
HMEC	human microvascular endothelial cell
HRE	hypoxia response element
HUVEC	human umbilical vein endothelial cell
IGF-1	insulin-like growth factor-1
IL	interleukin
IL-11R	interleukin 11 receptor
MAPK	mitogen-activated protein kinase
MetAP	methionine aminopeptidase
MMP	matrix metalloproteinase
MT-MMP	membrane-type matrix metalloproteinase
NG-2	high-molecular weight melanoma associated antigen
NRP-1	neuropilin-1
P	postnatal day
PDGF-B	platelet-derived growth factor B
PDGFR-β	platelet-derived growth factor receptor β
PEDF	pigment epithelium-derived factor
PI3K	phosphatidylinositol 3-kinase
PlGF	placental growth factor
PSMA	prostate-specific membrane antigen
SAGE	serial analysis of gene expression
S1P	sphingosine-1-phosphate
$S1P_1$	sphingosine-1-phosphate receptor
TβR	transforming growth factor β receptor
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor-α
TSP	thrombospondin
TU	transducing units
VE-cadherin	vascular endothelial cadherin

VE-statin	vascular endothelial statin
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	Von Hippel-Lindau
vWF	von Willebrad factor

Abstract

The inner surfaces of blood vessels are covered with heterogeneous population of endothelial cells. Phenotypes of these cells vary between different organs, between different parts of the vasculature in a given organ and even within neighboring endothelial cells of the same organ and the same type of blood vessel. Every single endothelial cell of the body is subjected to an infinite amount of signals leading to diverse phenotypes, including soluble factors, such as growth factors and chemokines, cell-cell and cell-basement membrane interactions and other variables, such as pH, pO_2, sheer stress from blood flow, stretch and temperature, to name a few. All these variables in the endothelial cell microenvironment will influence the phenotype--thus function--of the cell, to the extent that its predetermined genetic makeup allows. All these phenotypic and functional differences in endothelial cells lead to vascular heterogeneity, i.e. the organ-, tissue-, and vessel specific differences.

Structural and functional heterogeneity of the endothelium has been a subject for studies for decades, but only more recently has the focus of studies in endothelial cell biology shifted to the molecular heterogeneity of the endothelium. The exploration of the molecular diversity of blood vessels is a rapidly expanding research area that is driven by the vast potential of the discoveries in molecular heterogeneity to contribute to the development of targeted diagnostics and therapeutics. It is now recognized that a complex system of ligand-receptor pairs exists within tissues. The expression levels and activation states of these addresses are modulated in blood vessels during tumor progression, and can also be altered in the context of other pathological conditions involving abnormal blood vessel development and function, such as retinopathies, inflammation and atherosclerosis.

Our studies have demonstrated new roles for two aminopeptidases, CD13/ aminopeptidase N (CD13/APN) and aminopeptidase A (APA), in pathological angiogenesis. We show that these membrane-associated proteases are upregulated in angiogenic vasculature of murine and human tumors. By using genetic elimination and biochemical inhibition of CD13/APN and APA our results demonstrate an unrecognized mechanistic role for CD13/APN and APA in pathological angiogenesis. Thus, specific CD13/APN and APA inhibitors--such as chemical inhibitors, peptidomimetics or inhibitory anti-CD13/APN and anti-APA antibodies--may prove useful for translational applications targeting tumor vasculature. We also studied the regulation of *CD13/APN* gene expression by factors contributing to angiogenic progression and showed that CD13/APN functions in the control of endothelial cell morphogenesis and that endogenous CD13/APN levels in primary cells and cell lines are upregulated in response to hypoxia, angiogenic growth factors, and signals regulating capillary tube formation during angiogenesis. In addition, we showed that the proximal promoter of *CD13/APN* contains the essential elements for the angiogenic induction of *CD13/APN* expression *in vitro* and *in vivo*.

We also demonstrate that the angiogenic response in the retina can be altered by the reduction of the number of photoreceptor cells in the retina. Both mouse and human data indicate that reactive retinal neovascularization either fails to develop or regresses when the number of photoreceptor cells is markedly reduced. Our findings support the hypothesis that a functional mechanism underlying this anti-angiogenic state is failure of the predicted upregulation of vascular endothelial growth factor (VEGF), although other growth factors may also be involved in this complex biological phenomenon. These findings lead us to a hypothesis that reducing the metabolic rate of rod cells at critical time windows may improve the incidence of retinopathy of prematurity or perhaps slow the progression of diabetic retinopathy in adults.

Finally, we developed new methodology for the identification, validation, and prioritization of functional molecular targets on endothelial cells and human blood vessels. Biopanning and rapid analysis of selective interactive ligands (termed BRASIL) is based on single-step organic phase separation and it is faster, more sensitive and more specific than current methods. BRASIL may prove itself as a superior method for probing target cell surfaces with a broad range of potential applications. We also took the first steps towards the assembly of ligand-receptor based molecular map of the blood vessels in the human body; the vascular map, by performing an *in vivo* screening of a peptide library in a patient. A survey of 47,160 motifs that localized to different organs indicates that the tissue distribution of circulating peptides is nonrandom. High-throughput analysis of the motifs revealed similarities to ligands for differentially expressed cell-surface proteins, and a candidate ligand–receptor pair was validated.

Identification of vascular bed specific ligand-receptor pairs and knowledge about their cellular distribution and accessibility will be requisite for the development of endothelium-targeted therapies. In addition, many changes in the expression patterns of cell surface molecules during pathological blood vessel formation reflect the various activities of those molecules that are required during the formation of new blood vessels. Modulating the activities of these molecules may lead to inhibition or even regression of new blood vessel formation offering an important therapeutic opportunity for pathological conditions involving angiogenesis.

Review of the literature

1. Components of the vasculature

1.1. Endothelial cells

The endothelium, which lines the blood vessels, is a truly pervasive cell layer composed of 1-6 x 10^{13} cells covering a 1-7 m^2 surface area in human. The endothelium is highly active, participating in several physiological processes. Endothelial cells are elongated and thin cells lining the inner lumen of the blood vessel walls. They do not only provide a functional barrier between the circulation and surrounding tissue, but they also secrete mediators that affect vascular hemodynamics. Endothelial cells secrete vasodilators such as nitric oxide and prostacyclin and vasoconstrictors such as endothelin and platelet-activating factor regulating blood pressure and blood flow. Endothelium also facilitates the blood flow by creating an antithrombotic surface inhibiting platelet aggregation and blood clotting, but after vascular damage the endothelium changes to a prothrombic surface facilitating fibrin clot formation. Endothelial cells express surface molecules that control the trafficking of circulating cells (reviewed in Cines *et al.*, 1998). Finally, endothelial cells have an important role in angiogenesis, the formation of new blood vessels from existing ones, which accompany many pathological conditions including inflammation, diabetic retinopathy and cancer (reviewed in Carmeliet, 2003).

In the embryo, endothelial cells originate from hemangioblasts (Choi *et al.*, 1998) present in various organs (Jiang *et al.*, 2002) and in the adult, from endothelial progenitor cells, mesoangioblasts, multipotent adult progenitor cells or side population cells in the bone marrow (Reyes *et al.*, 2002). Endothelial cells can originate also by the migration and proliferation of endothelial cells of existing vessels (angiogenesis; reviewed in Carmeliet, 2003). Endothelial cells have half-lives of several years, but when stimulated, they are capable of rapid proliferation and migration, and sending out sprouts in a coordinated manner.

One of the important determinants of endothelial cell differentiation is the local microenvironment where they reside, and especially the interactions with surrounding cells. Endothelial cells communicate between surrounding endothelial cells, periendothelial cells/mural cells (such as smooth muscle cells and pericytes) and cells inside the vessel lumen, and dynamically interact with these cells and their surrounding extracellular matrix.

There are several useful markers for the identification of endothelial cells. Vascular endothelial cadherin (VE-cadherin; Lampugnani *et al.*, 1992) and vascular endothelial growth factor (VEGF) receptors (Peters *et al.*, 1993) are more specific to vascular endothelial cells, whereas angiotensin converting enzyme (ACE: Belloni and Tressler, 1990) von Willebrad factor (vWF; Yamamoto *et al.*, 1998), platelet-endothelial cell adhesion molecule PECAM (CD31; Vecchi *et al.*, 1994), P-selectin (Johnston *et al.*, 1989) and CD34 (Fina *et al.*, 1990) are also present in other (predominantly hematopoietic) cell types. Uptake of acetylated low density lipoprotein has been widely used as a marker of endothelial cells (Voyta *et al.*, 1984). Inducible expression of cell adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and E-selectin by inflammatory cytokines can be used as a marker to identify endothelial cells, particularly postcapillary venule-derived endothelial cells (Bevilacqua, 1993). There are many more endothelial cell markers than mentioned here, and I will refer the reader to *Aird (2003),* for a more complete list of markers.

Endothelial cells are plastic with respect to their proliferative capacity and differentiation potential. Endothelial cells can differentiate into either arterial or venous endothelial cells during embryonic development, neonatal retina and even in the heart of an adult (Stalmans *et al.*, 2002; Visconti *et al.*, 2002). Growth factor induced endothelial cells have the inherent ability to form a network of capillaries *in vitro* (Flamme and Risau, 1992). A vast number of growth factors and growth inhibitors influence the phenotype and functions of endothelial cell through the signaling of their receptors on endothelial cells including vascular endothelial cell growth factors (VEGFs), angiopoietins 1 and 2 (ang-1 and ang-2) transforming growth factor β (TGF-β) and Ephrin B2 to name a few (these growth factors and their receptors will be reviewed and referenced throughout this review of the literature). Endothelial cells communicate with other endothelial cells and periendothelial cells/mural cells through adherens and gap junctions and through tight junctions in brain and retinal capillaries. VE-cadherin mediates the adherens type endothelial-endothelial cell junctions in blood vessels (Lampugnani *et al.*, 1992) and controls an endothelial cell survival pathway through its intracellular interactions with β-catenin (Carmeliet *et al.*, 1999). Adherens type endothelial-mural cell junctions are mediated by VE-cadherin and N-cadherin. The α_5 connexin/connexin 40 and α_4 connexin/connexin 37 are expressed in endothelial cells and form the gap junctions between endothelial cells and between endothelial cells and mural cells. Tight junctions in brain and retinal vasculature regulating interactions between endothelial cells and mural cells are formed by occludins, zona occludins and claudins (reviewed in Darland and D'Amore, 2001). Cell surface adhesion molecules integrins govern interactions and communication between endothelial cells (as well as mural cells) and the matrix surrounding these cells. Of the more than 20 known integrins, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are known to be expressed on endothelial cells and $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_8\beta_1$, $\alpha_9\beta_1$, $\alpha_v\beta_1$, $\alpha_6\beta_4$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on mural cells (the role of integrins

in vascular control is reviewed in Martinez-Lemus *et al.*, 2003).

1.2. Periendothelial cells/mural cells

In addition to endothelial cells, periendothelial cells or mural cells (pericytes in capillaries and postcapillary venules and smooth muscle cells in larger vessels) are also required for the formation of functional blood vessels.

Pericytes are adventitial cells lying within the basement membrane of capillaries and postcapillary venules at the interface between the endothelium and the surrounding tissue. They are functionally codependent on endothelial cells, each cell type influencing each other's phenotypic expression and function. Initially, pericytes were recognized by their morphology and their location adjacent to endothelial cells within the same basement membrane (reviewed in Allt and Lawrenson, 2001). Nowadays the more common way to identify pericytes is by the expression patterns of several molecular markers. Such markers include α-smooth muscle actin (α-SMA), non muscle myosin, tropomyosin, desmin, nestin, platelet-derived growth factor receptor β (PDGFR-β) and high-molecular weight melanoma-associated antigen (NG2). Identification of pericytes is not straightforward since the marker expression varies between different organs and vessel types (Morikawa *et al.*, 2002).

Periendothelial cells originate from multiple sources during embryonic development, including the local and distal mesenchyme (reviewed in Hungerford and Little, 1999 and in Sartore *et al.*, 2001). Some evidence for transdifferentiation of embryonic endothelial cells to smooth muscle cells has been reported *in vivo*. Endothelial cells from the dorsal aorta can also transdifferentiate into periendothelial cells (DeRuiter *et al.*, 1997). Both hemangioblasts and angioblasts may have some potential to also generate smooth muscle cells (Ema and Rossant, 2003). VEGF receptor (VEGFR)-2 positive cells derived from embryonic stem cells can differentiate into both endothelial cells and mural cells (pericytes and vascular smooth muscle cells) and can reproduce the vascular organization process. With VEGF treatment, VEGFR-2 positive embryonic stem cells form endothelial cells sheets whereas in the absence of VEGF, expression of VEGFR-2 is lost, and cells differentiate into mural cells through mainly platelet-derived growth factor-B (PDGF-B) signaling (Yamashita *et al.*, 2000). In adult, putative smooth muscle progenitor cells have been isolated from human peripheral blood and further differentiated to smooth muscle outgrowth cells *in vitro* (Simper *et al.*, 2002) and to smooth muscle cells *in vivo* (Yeh *et al.*, 2003). Fibroblasts can also differentiate into myofibroblasts, which in turn differentiate into vascular smooth muscle cells in response to biochemical or mechanical cues (Tomasek *et al.*, 2002; Chambers *et al.*, 2003).

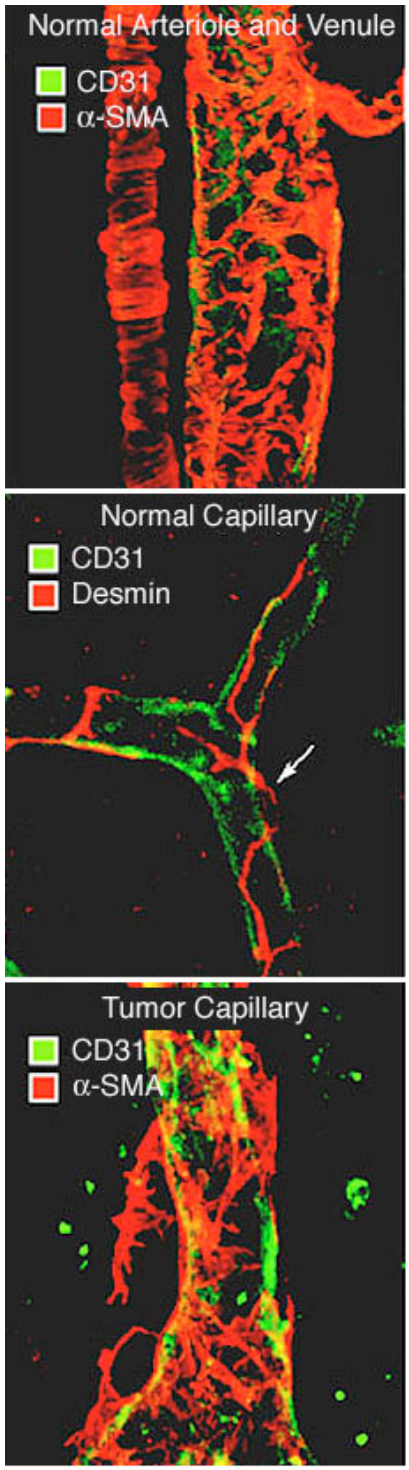

Figure 1. Morphology of mural cells on normal and tumor blood vessels. Tissue sections from normal and tumor tissues were stained for CD31 (green) and α-SMA or desmin (red) in order to identify endothelial cells and mural cells, respectively. Upper panel: Smooth muscle cells are circumferentially oriented and closely spaced on a larger arteriole (left) and venule (right) in normal tissue (pancreas). Middle panel: Arrow is pointing to a pericyte positioned along the vessel axis on normal capillary. Most of the pericyte processes are oriented longitudinally. Lower panel: Unlike normal pericytes some pericytes in tumor blood vessels project away from the endothelium and into the tumor parenchyma and some pericytes in overlap other pericytes. Adapted with permission, from Morikawa *et al.*, 2002.

Pericytes significantly determine the development of blood vessels. They may act in a paracrine manner and through cell-cell dependent contacts with endothelial cells. During the development of blood vessels, endothelial cells direct the migration of undifferentiated mesenchymal cells and induce their differentiation toward a smooth muscle cell/pericyte lineage. Modulation of the proliferation and differentiation of mesenchymal cells occurs during the recruitment of these cells by endothelial cells. Heterotypic cell-cell contacts and soluble factors play a role in growth control during vessel assembly (Hirschi *et al.*, 1999). Pericytes stabilize nascent blood vessels by inhibiting proliferation and migration of endothelial cells, and they induce endothelial cell differentiation, quiescence and survival. Periendothelial cells also influence endothelial permeability. Periendothelial cells secrete extracellular matrix proteins and elastin fibers thus playing a role in establishing the structural integrity of the vessel wall as well as in vasomotion and viscoelasticity. They control vessel remodeling and plasticity, since smooth muscle cell covered blood vessels are more resistant to regression (reviewed in Allt and Lawrenson, 2001; Hellstrom *et al.*, 2001).

Endothelial cells produce chemotactic, growth and survival factors for mural cells, recruit the mural cells to the newly formed vessel and induce their differentiation. Most studied growth factors influencing mural cells are PDGF-B, TGF-β and angiopoietins (these growth factors and their receptors will be reviewed and referenced throughout this review of the literature). PDGF-B-PDGF receptor-β (PDGFR-β) ligand-receptor pair has a major influence on the pericyte phenotype *in vivo*. Endothelial cells secrete PDGF-B which induces the differentiation of PDGFR-β positive cells to mural cells (Hellstrom *et al.*, 1999); this ligand-receptor interaction is crucial for the establishment of the blood vessel wall. TGF-β induces differentiation of mesenchymal cells to myofibroblasts and smooth muscle cells (Chambers *et al.*, 2003). The endothelial TGF-β binding protein, endoglin, and its downstream signaling molecule Smad-5 also have essential role in vascular smooth muscle cell differentiation and their recruitment to blood vessel wall (Li *et al.*, 1999; Yang *et al.*, 1999). Ang-1 through the interactions with its receptor Tie2 stabilizes blood vessels by facilitating communication between endothelial cell and mural cells (Suri *et al.*, 1996). *In vitro* Ang-1 enhances endothelial cell stimulated smooth muscle cell migration by a mechanism involving up-regulation of heparin binding EGF-like growth factor (HB-EGF) expression in endothelial cells and subsequent signaling via ErbB1 and ErbB2 in smooth muscle cells and pericytes (Iivanainen *et al.*, 2003).

Rather low density of pericyte coverage is required for basal microvascular function, since up to 90% reduction of pericyte coverage is still tolerated during embryonic development and postnatal survival, while loss of 95% of the pericyte coverage is lethal (Lindahl *et al.*, 1997; Enge *et al.*, 2002). Current anti-angiogenic approaches have been focusing mainly on endothelial cells, but recent studies suggest that combinatorial strategies targeting both the endothelial and perivascular cell compartments are likely to provide improved efficacy for anti-angiogenic therapies in multiple stages of tumorigenesis (Bergers *et al.*, 2003).

Although several growth factors and chemotactic factors have already been described for the proliferation and recruitment of vascular smooth muscle cells around capillaries, only a few repressors of vascular smooth muscle cell migration is known thus far. Endothelial cells produce an inhibitory protein of mural cell migration, called vascular endothelial-statin (VE-statin; Soncin *et al.*, 2003). Secreted VE-statin inhibits PDGF-BB -induced smooth muscle cell migration, but not proliferation, without affecting endothelial cell migration. Unlike many other endothelial cell markers, VE-statin is expressed by endothelial cells regardless of their origin (vein or artery) or vessel size (Soncin *et al.*, 2003). The receptor and intracellular signaling pathways of VE-statin in vascular smooth muscle cells remains to be identified.

1.3. Basement membrane

Cells in tissues are surrounded by the extracellular matrix (ECM), which is composed of long and filamentous protein fibers providing tensile strength and channels for communication and movement of cells within the tissue. The main proteinaceous components of the ECM are the structural proteins, collagens, produced by a variety of stromal cells, mainly fibroblasts. ECM contains also proteoglycans such as lecticans and heparan sulphate proteoglycans (including perlecan), latent and active growth factors as well as matricellular proteins, such as SPARC, thrombospondins-1 and -2 (TSP-1 and TSP-2), osteopontin, tenascins-C and -X and hevin/SC1 (reviewed in Bosman and Stamenkovic, 2003).

The basement membrane is a specialized form of ECM that separates epithelium from the stroma of the tissue. The basement membrane is also an important component of blood vessels; it forms the scaffold for the endothelial cells and pericytes, the inner lining of this cylinder being lined by the endothelial cells and pericytes covering the outer part of the cylinder. The cells producing and secreting the proteins of the basement membrane are the very same cells, endothelial cells and pericytes, which it envelops. The main constituents of the basement membrane include type IV collagen, laminin, heparan sulphate proteoglycans and nidogen. The basic structure of the basement membrane is formed by the collagen network/scaffold, but another scaffold, the laminin scaffold, forms independently interacting with the collagen scaffold through nidogen (Aumailley *et al.*, 1989; Timpl and Brown, 1996). Other adhesive glycoproteins, like tenascin, as well as proteoglycans adhere to the scaffold and interact with cells in or adjacent to the matrix.

Matricellular proteins have an important role in modulating cell-matrix interactions. Several different isoforms of type IV collagen, laminin, proteoglycans and nidogen have been described providing specificity to the basement membrane associated with different tissue types. Tissue specific functions are the consequence of the different compositions of the basement membrane resulting in the regulation of the tissue specific behavior of endothelial cells (reviewed in Kalluri, 2003).

Basement membranes and other ECMs undergo constant dynamic changes in response to a vast number of cellular stimuli, and the remodeling of the vascular basement membrane is a vital aspect of angiogenesis. When endothelial cells and mural cells migrate to form new blood vessels, the ECM network is not only degraded, but its composition is also altered and new cryptic epitopes of the ECM proteins are exposed that facilitate the migration of endothelial and mural cells. Growth factor stimulation leads to the secretion of ECM digesting enzymes from endothelial cells, and in some cases from the surrounding stromal cells and tumor cells (reviewed in Kalluri, 2003).

Besides providing structural framework upon which cells grow, migrate and differentiate to form blood vessels, the basement membrane is an important source of angiogenic growth factors, such as basic fibroblast growth factor (bFGF), VEGF, PDGF-B and TGF-β as well as angiogenesis inhibitors, such as TSP-1, canstatin, tumstatin, platelet factor-4 and endostatin. Cell produced proteinases are required for the liberation of these factors from the matrix (reviewed in Egeblad and Werb, 2002, and Kalluri, 2003). ECM-bound VEGF can also promote endothelial cell adhesion, migration, and survival through integrin ligation. A new VEGF receptor-independent role for immobilized VEGF in supporting cell adhesion and survival has been discovered to act through interactions with integrins. Immobilized VEGF almost totally abolishes endothelial cell apoptosis through interactions with integrins (Hutchings *et al.*, 2003).

Degradation and remodeling of the ECM must occur in a balanced manner, thus, it is a highly regulated process. Insufficient breakdown of the ECM will prevent endothelial and mural cells from leaving their original position, but on the other hand, excessive breakdown of the ECM removes critical support and guidance cues for migrating endothelial and mural cells inhibiting the formation of new blood vessels. Studies with matrix metalloproteinase (MMP) inhibitor RECK elegantly demonstrates the importance of the ECM degradation and regeneration for normal angiogenesis. In the absence or RECK expression, excess MMP activity results in increased degradation of the ECM, causing reduced structural integrity of blood vessels and surrounding tissue. Consequently, the maturation of the early vascular network gets stalled. In the presence of excessive RECK expression, MMP activity is inhibited to the point where ECM remodeling is compromised and the pre-existing blood vessels are not able to send out capillary sprouts. Consequently, the formation of new blood vessels is inhibited (Oh *et al.*, 2001).

2. Development of the vasculature

Cardiovascular system is the first functional organ system to develop during embryogenesis. The failure of the cardiovascular system to develop will unavoidably lead to embryonic lethality. During vasculogenesis, the primary capillary plexus is formed by proliferating endothelial precursor cells, angioblasts, coalescing into a primitive vascular network (Risau and Flamme, 1995). This endothelial cell network further serves as a scaffold for angiogenesis, the sprouting, bridging and intussusceptive growth of existing blood vessels (Risau, 1997). The immature vascular network must mature at the level of the blood vessel wall as well as at the level of overall vascular system, forming a highly organized network of blood vessels providing all the parenchymal cells of the body the required nutrients and oxygen (see Jain, 2003, for an excellent review). VEGFs are probably the most critical drivers of vascular formation both during development and postnatal life regulating differentiation of angioblasts to endothelial cells, endothelial cell proliferation and fusion into tubes with lumina and organization into primitive plexus as well as in endothelial cell sprouting. VEGFs and their receptors will be discussed in more detail in section 3.3.

2.1. Formation of immature vasculature

The first step in the development of a functional vascular network is the formation of immature vasculature. The formation of the primary capillary plexus starts from blood islands, which are aggregations of mesodermal cells that colonize the yolk sac and where the earliest hematopoietic cells and endothelial cells appear. Peripheral cells, angioblasts, surrounding primitive erythrocytes differentiate into endothelial cells, whereas central cells differentiate into hematopoietic cells (Risau and Flamme, 1995). The first hematopoietic and endothelial progenitors are derived from a common embryonic precursor termed hemangioblast (Choi *et al.*, 1998). The faith of hemangioblasts are determined by co-expression of lineage-specific transcription factors. Recently, a homeoprotein HEX has been indicated as a regulator of hemangioblast differentiation into hematopoietic and endothelial cell lineages (Guo *et al.*, 2003), and the transcription factor Tal1/SCL has been shown to regulate the choice of cell fate into hematopoietic, smooth muscle and endothelial cell lineages (Ema *et al.*, 2003).

The endothelial cells lining the blood vessels differentiate from mesodermal CD31, CD34, VEGFR-2 positive precursor cells, angioblasts, to form a primitive vascular network in a process called vasculogenesis giving rise to the dorsal aorta, the cardinal vein and the embryonic stems

of yolk sac arteries and veins. Blood vessel formation is dependent on the action of VEGF on its receptor tyrosine kinases, VEGFR-1/Flt-1 and VEGFR-2/Flk-1, expressed in endothelial cells (Fong *et al*., 1995; Shalaby *et al*., 1995; Carmeliet *et al*., 1996; Ferrara *et al*., 1996). Deletion of *flk*-1 causes an arrest in vasculogenesis during the angioblast differentiation state (Shalaby *et al*., 1995), while deletion of *flt-1* causes embryonic lethality from edema following initial formation of definitive blood vessels (Fong *et al*., 1995). Current models for the genetic programming of vasculogenesis suggests that a multifactorial combinations of transcription factors in a tightly synchronized expression pattern are required for vasculogenesis (Oettgen, 2001).

Hypoxia is an important stimulus for the expansion of the vascular bed. Initially, cells in the developing embryo are oxygenated by diffusion of oxygen. Sprouting angiogenesis is probably triggered by hypoxia when the tissues grow beyond the limit of oxygen diffusion. Hypoxia is signaled through hypoxia-inducible transcription factors (HIFs) that induce the expression of number of genes involved in blood vessel formation and maturation, including VEGF, nitric oxide synthetase and Ang-2 (Semenza, 2003a). Blood vessels become leaky, and the plasma proteins leaked from these nascent vessels serve as a provisional matrix. The basement membrane is degraded by the activated proteases, such as MMPs (Egeblad and Werb, 2002). Differential expression of cell adhesion molecules like vascular cadherins (Lampugnani *et al*., 1992) and integrins (Friedlander *et al*., 1995) is also essential for the formation of nascent blood vessels, where growth factors and morphogens differentially regulate the expression of these molecules. Endothelial cells migrate through interactions between integrins and the ECM, and proliferate in response to endothelial cell mitogens such as VEGF.

2.2. Stabilization of immature vasculature

The signaling of Angiopoietins (ang 1-4) through their receptor tyrosine kinase system (Tie1 and Tie2), play an important role in the formation of the vascular loops and networks in the immature vasculature and the further stabilization of this immature vasculature. Tie1 and Tie2 are expressed in embryonic angioblasts, vascular endothelium and endocardium, whereas in adult, Tie1 is expressed in endocardium and lung capillaries and Tie2 is expressed in endocardium and vascular endothelium (Dumont *et al*., 1994; Korhonen *et al*., 1994). Genetic studies implicate a major role for the Tie receptor system in angiogenesis and vascular remodeling. In Tie1 deficient mice, blood vessels are established, but the vascular integrity is compromised leading to embryonic or perinatal lethality, suggesting a role for Tie1 in the integrity and survival of endothelial cells in angiogenesis. Tie2 is required for sprouting and branching of blood vessels during angiogenesis; Tie2 deficient mice have reduced number of endothelial cells and abnormal vascular network formation lacking the normal sprouting, branching and remodeling of blood vessels. Consequently, Tie2 deficiency is embryonically lethal (Dumont *et al*., 1994; Sato *et al*., 1995). Tie2 receptor ligand Ang-1 is critical for vascular development in stabilizing the forming blood vessels making them impermeable by facilitating the endothelial cell-mural cell communication. Ang-1 deficient mice have a similar phenotype as Tie2 deficient mice, including embryonic lethality (Suri *et al*., 1996). Ang-1 is expressed in embryonic heart myocardium (Suri *et al*., 1996) and later during development within the mesenchyme surrounding developing blood vessels. In adult, the main sources of Ang-1 are the mural cells; vascular smooth muscle cells and pericytes (Davis *et al*., 1996). Ang-1 has a role in pericyte recruitment to developing blood vessels by stimulating endothelial cells to produce growth factors that in turn stimulate the differentiation of the surrounding mesenchyme into pericytes or smooth muscle cells. In Ang-1 deficient mice, endothelial cells are poorly associated in the underlining basement membrane, and they do not recruit pericytes properly leading to more rounded endothelial cell phenotype (Suri *et al*., 1996). In contrast, mice over expressing Ang-1 show increased vascularization (Suri *et al*., 1998), and in the absence of mural cells, exogenous Ang-1 can restore the hierarchal order of functionality of larger blood vessels in developing retinal vasculature in mice (Uemura *et al*., 2002). Ang-1 is involved in regulating endothelial cell survival, endothelial cell sprouting and blood vessel branching *in vitro* and *in vivo* as well as in regulating endothelial cell-basement membrane interactions by serving as a substrate for cell adhesion through integrins and Tie2 (Carlson *et al*., 2001). In addition of being secreted, Ang-1 is incorporated in the ECM of tumor cells. Endothelial cell adhesion causes a release of ECM associated Ang-1 and subsequent Tie2 receptor activation (Xu and Yu, 2001). Ang-1 and Tie1 in combination are critical in establishing vascular polarity during angiogenesis since mice deficient for both Ang-1 and Tie1 showed a specific disruption of the right system of the sinus venosus (Loughna and Sato, 2001).

Ang-2 plays a dynamic role in vascular remodeling and angiogenesis in conjunction with VEGF and Ang1. In the absence of VEGF, Ang-2 acts as an antagonist of Ang-1 and destabilizes blood vessels, leading to vessel regression whereas in the presence of VEGF, Ang-2 facilitates vascular sprouting and branching by blocking the constitutive stabilizing Ang-1 signal (Maisonpierre *et al*., 1997). Ang-1 and TGF-β, both important in maturation and stabilization of developing blood vessels, downregulate Ang-2 expression, whereas for instance hypoxia and VEGF up-regulate the Ang-2 expression (Mandriota and Pepper, 1998). Angiotensin II (AngII) induces Ang-2 expression without affecting Ang-1 or Tie2 expression. Furthermore, angiotensin receptors 1 and 2 (AT1 and AT2) regulate Ang-2 and VEGF expression

differentially. AT1 stimulates the ECM bound EGF by metalloproteinases, which transactivates EGF-receptor to induce angiogenesis via the combined effects of Ang-2 and VEGF. On the other hand, AT2 attenuates these affects by blocking EGF-receptor phosphorylation (Fujiyama *et al.*, 2001).

TGF-β and its signaling pathways play a role both in the formation and stabilization of the vasculature. TGF-β induces differentiation of endothelial cells after their recruitment to endothelial walls (Hirschi *et al.*, 1998) and through endoglin signaling, functions in the recruitment of mesenchymal cells to blood vessels and their differentiation to mural cells (Li *et al.* 1999).

Paracrine signaling via PDGF-B expressed in endothelial cells and its receptor PDGFR-β expressed on mural cells plays an important role in mural cell recruitment to blood vessels and the following stabilization of the vessel. PDGF-B and PDGFR-β deficient mice lack pericytes and develop microaneurysms and die late in gestation (Lindahl *et al.*, 1997). Pericyte deficiency leads to a variety of microvascular abnormalities including endothelial cell hyperplasia and abnormalities in shape and ultrastucture, vessel tortuosity and leakiness leading to widespread microaneurysms (Hellstrom *et al.*, 1999; Hellstrom *et al.*, 2001). This reduced coverage of dilated and tortuous microvessels by pericytes leads to embryonic lethality due to fatal hemorrhages in both PDGF-B knockout mice (Leveen *et al.*, 1994; Lindahl *et al.*, 1997) and PDGFR-β knockout mice (Soriano, 1994; Lindahl *et al.*, 1997).

Sphingosine-1-phosphate (S1P)- sphingosine-1-phosphate receptor ($S1P_1$/Edg1) interaction is also essential for blood vessel maturation (Liu *et al.*, 2000). $S1P_1$ is a widely distributed G-protein coupled receptor for S1P, a bioactive platelet-derived lipid (Zhang *et al.*, 1991). Disruption of the *edg-1* gene results in massive embryonic hemorrhage and embryonic lethality caused by an incomplete vascular maturation due to a failure in vascular smooth muscle cell/pericyte recruitment to immature blood vessels (Liu *et al.*, 2000). This recruitment of vascular smooth muscle cells/pericytes is directed by the activity of the $S1P_1$ receptor in endothelial cells even though both endothelial cells and vascular smooth muscle cells/pericytes express S1P1 receptor (Allende *et al.*, 2003). Loss of $S1P_1$/Edg1 results in dysfunctional migration of diverse cell types (including aortic smooth muscle cells) toward PDGF (Hobson *et al.*, 2001), linking the phenotype of *edg-1* knockout mice (Liu *et al.*, 2000) to the phenotypes of *PDGF-BB* and *PDGFR-β* phenotypes. This highlights the importance of cross-communication between PDGFR and $S1P_1$/Edg1 in which activation of the G-protein coupled receptor, $S1P_1$/Edg1, by a receptor tyrosine kinase, PDGFR-β, is critical for cell motility playing an essential role in the maturation of the vasculature (Hobson *et al.*, 2001).

The currently favored view states that the initial endothelial tubes form without pericyte contact, ant that subsequent acquisition of pericyte coverage leads to vessel remodeling, maturation and stabilization. Improved means of identifying and visualizing pericytes now challenge this view and show that high number of pericytes invest an actively sprouting and remodeling vessels (Morikawa *et al.*, 2002).

2.3. Branching, remodeling and pruning of vasculature

The growth, branching, remodeling and pruning of the different segments of the developing vascular network determine the hierarchy of the vascular network with an optimal pattern for a given tissue. There are several mechanisms that can result in the branching of blood vessels. New vessel branches can sprout towards a cluster of cells in the surrounding mesenchyme that produces the angiogenic stimulus. Existing vessels can split into individual daughter vessels by the formation of transendothelial cell bridges or the vessels can branch via intussusception, based upon insertion of interstitial tissue columns into the lumen of pre-existing vessels (Djonov *et al.*, 2000). The subsequent growth of these columns and their stabilization results in partitioning of the vessel lumen and remodeling of the vascular network.

The spatial distribution of secreted VEGF-A is critical for the balance between capillary branching and growth in vessel size since mice lacking heparin-binding VEGF-A isoforms and therefore ECM interaction domains, exhibited a specific decrease in capillary branch formation and impairment of the direct extension of endothelial cell filopodia (Ruhrberg *et al.*, 2002). The patterning of retinal blood vessels has also been shown to depend on the balance between two different qualities of the extracellular VEGF-A; the gradient and the concentration. Properly shaped extracellular pattern of VEGF-A distribution is necessary for the correct guidance of filopodial extension from specialized endothelial cells at the tips of the vascular sprouts, and endothelial cell proliferation occurs in the sprout stalks in response to VEGF-A concentration. Both of these effects are mediated via VEGFR-2 (Gerhardt *et al.*, 2003). VEGFR-1 has recently been suggested also to play a role in vascular sprout formation and morphogenesis. VEGFR-1 was shown to positively affect sprout formation by negatively controlling the amount of VEGF-A signal that is sensed by endothelial cells, using soluble VEGFR-1 as the primary mediator of the effect (Kearney *et al.*, 2004). Kearney *et al.* also suggests a critical role for VEGF-A and/or VEGFR-1 gradient formation in modulating vascular sprout formation. Thus, the local availability of VEGF-A signal is critical to proper vascular morphogenesis and sprouting (Gerhardt *et al.*, 2003; Kearney *et al.*, 2004). In addition to signaling through VEGFRs, VEGF also signals through neuropilin receptors (reviewed in Neufeld

et al., 2002). VE-cadherin is essential for normal vascular integrity and the expansion and remodeling of the primitive vascular network, but it is not required for the initial assembly of the vascular endothelium (Carmeliet *et al*., 1999). VE-cadherin controls an endothelial cell survival pathway through its intracellular interactions with β-catenin (Carmeliet *et al*., 1999). VE-cadherin deficient endothelial cells are unable to respond to VEGF-A dependent cell survival signals through formation of a VE-cadherin/β-catenin/phosphatidylinositol 3-kinase (PI3K)/VEGFR-2 complex. In mice, genetic ablation of *VE-cadherin* or VE-cadherin-β-catenin interaction impair expansion, remodeling and maturation of vascular network and reduced endothelial cell survival causing embryonic lethality (Carmeliet *et al*., 1999). Neuropilin-1 and –2 (NRP-1 and NRP-2) belong to the class-3 semaphorin subfamily and function as receptors for the axon guidance factors during embryonic development. During blood vessel formation, neuropilins regulate VEGFR-1 and -2 signal transduction by forming complexes with these receptors. Besides disorganization of nerve fibers, NRP-1 deficient mice showed defects in neural vascularization, transposition of blood vessels and development of vascular network of the yolk sac leading to embryonic lethality (reviewed in Neufeld *et al*., 2002). Recently, semaphorin-3, a NRP-2 receptor ligand was shown to inhibit tumor angiogenesis (Kessler *et al*., 2004).

Nerves often run along larger blood vessels in adult tissues. The intimate association of nerves and blood vessels and their functional interactions, like the control of vasoconstriction and vasodilation, has been the reason to speculate that the branching patterns of these two systems might be ontogenetically or mechanistically related. Arteries are preferentially aligned with nerves following their branching pattern, whereas veins are not. Arteries fail to develop properly in mouse embryos that lack sensory nerves, while their branching is altered to follow the nerve in mouse embryos containing disorganized nerves. Peripheral nerves seem to provide a template determining the organotypic pattern of blood vessel branching and arterial differentiation, at least in the mouse skin, through local secretion of VEGF from sensory nerve fibers, Schwann cells surrounding them, or from both cell types (Mukouyama *et al*., 2002).

Components of the basement membrane and ECM provides cues for the growth and remodeling of the vasculature by regulating the proliferation, migration, survival and differentiation of endothelial cells, pericytes and smooth muscle cells. Degradation of the ECM involves an array of proteinases, which not only provide space for the migrating endothelial cells of the growing vessel, but also results in the release of matrix sequestered growth factors and proenzymes. In addition, degradation of the ECM can result in the exposure of cryptic adhesion sites hidden in non-proteolyzed matrix (reviewed in Kalluri, 2003). Endothelial cell movement through ECM is a tightly regulated process that requires the polarization of the molecular machinery for matrix degradation to the advancing edge of the moving cell, integrin mediated cell-matrix adhesion complex formation and subsequent disassembly involving reversible integrin/matrix binding, assembly and disassembly of cell cytoskeleton. In this process, integrins do not just mediate cell-cell and cell-matrix adhesion, but they also act as a link between the extracellular and intracellular environments allowing inside-out and outside-in signal transduction modulating endothelial cell functions (reviewed in Martinez-Lemus *et al*., 2003). Cells express a combination of integrins on their cell surface, and this combination is constantly changing during vascular development, suggesting that specific combination of integrins are required as development of the vasculature proceeds.

2.4. Specialization of blood vessels

The specialization of the tissue- and organ-specific structure of the blood vessel network and the molecular specialization of the endothelial cells lining the vessel walls are the least understood processes of the blood vessel development.

Arterio-venous specialization of blood vessels is genetically determined by the Notch-Gridlock signaling pathway (Lawson *et al*., 2001; Zhong *et al*., 2001). Bidirectional ephrin-ephrin receptor signaling during the formation of the capillary plexus repels the arterial and venous sides guiding vascular branching (Wang *et al*., 1998).

Cell-cell adhesion in developing blood vessels is mediated through adherens junctions that are formed by transmembrane adhesive protein VE-cadherin in endothelial cell-endothelial cell junctions and N-cadhering in endothelial cell-mural cell junctions. VE-cadherins are organized in clusters at cell-cell contacts and connect through their cytoplasmic domain with a complex network of cytoskeletal proteins. As the vessel matures, more classical cell-cell junctions including tight junctions and gap junctions start forming, depending on the function of the particular vascular bed (reviewed in Dejana, 1996). The factors determining the organ-specific nature of the cell-cell junction formation remain unknown.

Development of the retinal vasculature provides some interesting examples of the specialization of the blood vessels. In the retina, the fovea is specialized for high-resolution vision. Within the central fovea, cone photoreceptors reach their highest numerical density, yet the fovea is avascular. During the development of the retinal vasculature, the slow growth of blood vessels towards the central fovea and the low levels of endothelial cell proliferation suggest a presence of anti-proliferative and/or anti-angiogenic factor(s) within the foveal region. The requirement for nutrients and oxygen by photoreceptor cells, which are metabolically extremely active, must be

met by the specialization of the extra-retinal choroidal circulation; in the vicinity of the fovea, the density of the choroidal arcades is increased (reviewed in Provis, 2001).

Minority of blood vessels that forms during embryonic development persists until adulthood. After the onset of circulation, the primary capillary plexus is remodeled many times until the mature vascular network is formed. For example, all capillaries in the prechondrogenic area regress to allow the differentiation of the cartilage tissue (Hallmann *et al*. 1987). Hyaloid vascular system of the developing eye regresses to allow the development of a translucent vitreous body compatible with vision (Latker and Kuwabara, 1981).

Specialization of the developing vasculature leads ultimately to a vast structural and molecular heterogeneity of the vasculature, which will be the topic of the section 4 of this review of the literature.

2.5. Development of lymphatic vessels

Lymphatic vessels collect extravasated immune cells as well as fluid and proteins leaked from blood vessels and return them back to the circulation after passing through a series of lymph nodes. Lymphatic vessels develop from specialized endothelial cells in pre-existing blood vessels. The homeobox gene Prox1 was identified to be required for the induction of lymphatic vascular development from preexisting embryonic veins (Wigle and Oliver, 1999) and has an essential role in the developmental program specifying lymphatic endothelial cell fate (Hong *et al*., 2002).

Expression of Prox1 is sufficient to induce a lymphatic phenotype in blood vessel endothelial cells. Ectopic expression of Prox1 downregulates blood vessel-associated genes and also upregulates some of the known lymphatic endothelial cell markers suggesting that the blood vascular phenotype represents the default in the endothelial cell differentiation and they identify an essential role of Prox1 in the program specifying lymphatic endothelial cell fate (Hong *et al*., 2002). Lymphatic endothelial cells might also be derived from mesenchymal precursor cells, lymphangioblasts (Papoutsi *et al*., 2001). VEGF-C has been shown to be the paracrine factor essential for lymphangiogenesis (Karkkainen *et al*., 2004). Pericytes have been though to play a lesser role in the lymphatic vascular bed, but lymphatic endothelium has been shown to recruit pericytes/smooth muscle cells *in vivo*, collecting lymphatic vessels being covered by smooth muscle cells in contrast to lymphatic capillaries, which are negative for α-SMA positive cells (Veikkola *et al*., 2003).

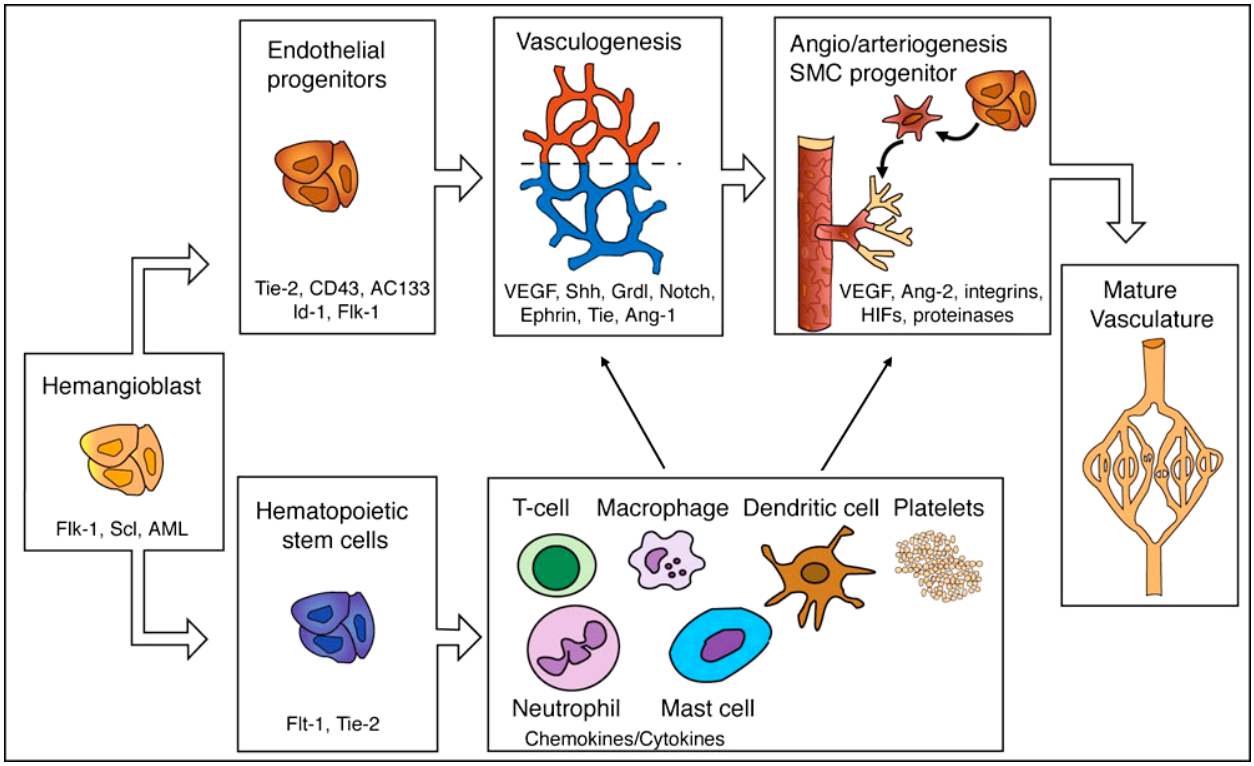

Figure 2. Formation of a vascular network. Endothelial progenitors differentiate to arterious and venous endothelial cells, which assemble in a primitive capillary plexus. Vessels then sprout and become stabilized by smooth muscle cells and pericytes, differentiating from their progenitors. Hematopoietic stem cells contribute to angiogenesis directly and indirectly, by differentiating to leukocytes or platelets. Shh, Sonic hedgehog; Grdl, gridlock, AML, acute myeloid leukemia; Scl, stem cell leukemia. Adapted from Carmeliet, 2003.

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3. Angiogenesis

3.1. Formation of angiogenic vasculature

Angiogenesis occurs during embryonic development and it is an essential process for the development of a functional vascular system. The failure of angiogenesis to occur during development will unavoidably lead to the death of the embryo. In adult, angiogenesis is involved in tissue repair, female reproductive cycle and inflammation. Angiogenesis also characterizes several pathological conditions including cancer and retinopathies (Carmeliet, 2003).

During cancer progression, in order for cells within an aberrant proliferative lesion to develop to a larger size into a tumor, cells must acquire angiogenic ability. The ability to induce and maintain angiogenesis is acquired in a discrete event during tumor development through an "angiogenic switch" from quiescent to activated vasculature (Hanahan and Folkman, 1996). The induction of angiogenesis is prerequisite for the rapid clonal expansion of tumor cells associated with the formation of macroscopic, clinically relevant tumors.

Counterbalancing positive and negative vascular growth signals induce or inhibit angiogenesis. Tumors activate the angiogenic switch by changing the balance of these growth signals. One strategy to do that involves altered expression of genes coding for vascular growth factors and inhibitors. Many tumors upregulate the expression of VEGF and FGFs and/or downregulate the expression of endogenous angiogenesis inhibitors including TSP-1. Altered expression or activation of proteases also play and important part in the angiogenic switch by controlling the bioavailability of angiogenic growth factors and inhibitors. A variety of proteases can release bFGF and VEGF from the ECM, while a component of the blood clotting system plasmin, and autocatalyze its own degradation from a pro-angiogenic molecule to an angiogenesis inhibitor, angiostatin (reviewed in Hanahan and Weinberg, 2000). Comparison of the gene expression patterns in endothelial cells from malignant versus non-malignant tissues by serial analysis of gene expression (SAGE) revealed that largest group of differentially expressed genes were genes coding for ECM proteins highlighting the role of the ECM and the endothelial cell/mural cell basement membrane and its functional regulation during angiogenesis (St Croix *et al*., 2000). Basement membrane does not disappear during angiogenesis, but instead remodels constantly as endothelial cells sprout during blood vessel growth. In angiogenesis, the basement membrane shows structural abnormalities consistent with the dynamic nature of endothelial cells and pericytes during angiogenesis (Baluk *et al*., 2003).

In pathological angiogenesis, the formed blood vessels show many abnormalities at the molecular, structural and functional level. The cell and ECM composition is also abnormal (reviewed in section 4.3.). Reasons for these abnormalities are not known, but at least part of the abnormalities in structure and function of tumor blood vessels reflect lack of proper maturation of blood vessels in the tumor microenvironment (Gee *et al*., 2003). Given the abnormalities in structure and function of tumor blood vessels, it seems likely that blood vessels in normal and tumor tissue form by different mechanisms.

Dvorak (2003) describes elegantly the formation of angiogenic blood vessels in response to VEGF-A in mouse tissue. First abnormal blood vessel structures appear soon (time frame in hours) after the initiation of VEGF-A overexpression in mouse tissue arising from the enlargements of pre-existing microvessels, mainly from venules. These VEGFR-2 positive sinusoids, named "mother vessels", are large and hyperpermeable and have thin walls, serpentine structure and poor pericyte coverage. Mother vessels are formed by a multi step process involving digestion of the blood vessel basement membranes, detachment of pericytes and morphological changes in endothelial cells including spreading and thinning of the cells to cover an expanded surface area. Vesiculo-vacuolar organelles, cytoplasmic vesicles and vacuoles in endothelial cells, provide an intracellular storage for membrane that can be mobilized rapidly to the growing plasma membrane allowing the enlargement of endothelial cells. Many mother vessels develop into poorly organized vascular structures called glomeruloid bodies. Glomeruloid bodies are first recognized few days after VEGF-A overexpression as focal collections of large primitive cells in the mother vessel lining. As glomeruloid bodies expand, the large lumens of mother vessels start dividing into multiple small and tortuous channels. As VEGF-A expression in the tissue subsides, glomeruloid bodies resolve to normal appearing capillaries through apoptosis and reorganization of endothelial cells and pericytes (Dvorak, 2003). The detailed mechanisms by which different growth factors produced by tumors or by inflammatory cells produce the angiogenic response are likely to differ, leading to different phenotypes of angiogenic vasculature.

In addition to the sprouting and co-option of neighboring pre-existing vessels, angiogenesis during tumor growth and wound healing is also supported by the mobilization and incorporation of circulating endothelial progenitor cells (CEPs) to the growing blood vessels. CEPs are highly proliferative bone marrow-derived cells that upon recruitment to growing blood vessels will differentiate into endothelial cells. VEGF through interaction with its receptors VEGFR-2 and VEGFR-1 expressed on endothelial and hematopoietic stem cells promotes the co-mobilization of CEPs and hematopoietic cells from the bone marrow and recruitment of these cells into the sites of tissue injury and angiogenesis. The collaboration between recruited CEPs and hematopoietic cells facilitates the differentiation and integration of CEPs into the

growing vascular bed (reviewed in Rafii *et al*., 2002).

3.2. Role of hypoxia in angiogenesis

Within the tissues, the architecture of the vascular network brings the circulatory system with oxygen and nutrients to close contact with all cells. Local oxygen tension seems to be the critical signal for cells to detect vascular insufficiency. This has a profound effect on the vasculature, which responds to hypoxia through the induction of new blood vessel formation. The diffusion coefficient of oxygen within tissues is about 150-200 μm. The location of both dividing and apoptotic tumor cells within spontaneous and implanted murine tumor models has been shown to reflect the oxygen diffusion in tissues; tumor cells located less than 100 μm away from a blood vessel are viable while more distant tumor cells are undergoing apoptosis (Fidler *et al*., 2002).

Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator that functions as the master regulator of O_2 homeostasis in tissues. HIF-1 is a heterodimer that consists of the hypoxic response factor HIF-1α and the constitutively expressed aryl hydrocarbon receptor nuclear translocator (ARNT; HIF-1β). In the absence of oxygen, HIF-1 binds to hypoxia response elements (HREs) in the gene promoter regions, thereby activating the expression of a variety of hypoxia-response genes. In the presence of oxygen HIF-1α is bound to the tumor suppressor Von Hippel-Lindau (VHL) protein. Subsequently, HIF-1α becomes ubiquitylated and targeted to the proteasome for degradation. This O_2-dependent activity of HIF-1 depends on hydroxylation of the HIF-1α by a prolyl hydroxylase protein and by a FIH1-protein. Hydroxylation of a proline residue is required for the binding of VHL protein to HIF1-α and the hydroxylation of an asparagine residue is required for the binding of HIF-1α co-activators p300 and CBP. Under hypoxic conditions, O_2 becomes the limiting factor for these hydroxylation reactions ultimately leading to a decreased rate of HIF-1α degradation (reviewed in Semenza, 2003b).

HIF-1-targeted genes encode proteins that function in pathways that increase O_2 delivery and mediate adaptive responses to O_2 deprivation. These pathways include angiogenesis, glycolysis, growth factor signaling, cell immortalization, tissue invasion and metastasis, apoptosis and pH regulation. To date, more than 60 putative direct HIF-1 target genes have been identified (reviewed in Semenza, 2003b).

Besides being regulated by the tissue O_2 concentration, the major growth factor-stimulated signal transduction pathways have a role in HIF-1 regulation by inducing the expression of HIF1α. In contrast to hypoxia, growth factors and cytokines stimulate HIF-1α synthesis through activation of the PI3K or mitogen-activated protein kinase (MAPK) pathways. While hypoxia increases HIF-1α levels in all cell types, growth factor induced HIF-1α expression is dependent on the cell type (reviewed in Semenza, 2003b). HIF-1α was also demonstrated to act as a negative or positive factor in astrocytoma progression depending on the microenvironment in which the tumor grew. This observation lead to the notion that HIF-1α-regulated hypoxic response to the microenvironment is highly dependent on the local environment in which those tumors arise and involves both growth factor (VEGF)-dependent and -independent mechanisms (Blouw *et al*., 2003).

In human cancer cells, intratumoral hypoxia, growth factor expression and genetic alterations affecting signal transduction pathways lead to increased HIF-1 activity, which promotes angiogenesis, metabolic adaptation, and other critical aspects of tumor progression (reviewed in Semenza, 2003b).

Development of the retinal vasculature brings us a well-characterized example of hypoxia during the development of vasculature. Blood vessels enter the back of the embryonic eye at the eyecup stage and reach the vitreal surface via the choroidal fissure. This fissure closes around the developing optic nerve and the blood vessels close to the vitreal surface that supply the innermost part of the central retina. As the retina expands during and after the fetal period, the vessels branch and grow radially outward toward the retinal periphery (reviewed in Provis, 2001). The invading retinal vasculature is associated with astrocytes. Astrocytes lie in the avascular zone just a few hundred micrometers ahead of the radially spreading vessels, and are thought to stimulate and control the direction of vessel growth by local release of VEGF (Sandercoe *et al*., 1999). Low tissue O_2 concentration is the key regulator of VEGF production in the developing retinal vasculature Differentiated astrocytes lying just ahead of the invading vascular endothelium are critically sensitive to hypoxia and respond by upregulation of VEGF which in turn stimulates the endothelial cell proliferation at the vascular front (Pierce *et al*., 1995; Stone *et al*., 1995). In a similar way, Müller cells are though to regulate development of the secondary vascular plexus in the retina (reviewed in Provis, 2001). Once blood vessels mature to the stage at which they are invested with pericytes they are believed to loose responsiveness to VEGF (Benjamin *et al*., 1998). Another set of blood vessels supplies the choroid, just external to the pigment epithelial layer. The pigment epithelial layer itself and the entire length of the photoreceptor cells, from their synaptic endings in the outer plexiform layer, through their nuclei in the outer nuclear layer, to their specialized inner and outer segments close to the pigment epithelium are normally avascular (reviewed in Provis, 2001). In experimental or clinical contexts in which retinal hypoxia induces VEGF expression, new blood vessels will form either on the inner neural retina in young subjects or, in some older subjects, from the choroid across the pigment epithelium (Schlingemann and van Hinsbergh, 1997). In contrast, when VEGF basal

expression drops, endothelial cells undergo apoptosis (Alon *et al*., 1995) and retinal vasculature regresses resulting in a reduced retinal blood supply. On the other hand, hypoxic regulation of angiogenic factors such as VEGF, TGF-β (de Kozak *et al*., 1997; Ogata *et al*., 1997), growth hormone (GH), insulin-like growth factor-I (IGF-1) and its receptors (Smith *et al*., 1997), placental growth factor (PlGF; Khaliq *et al*., 1998) and inhibitory factors such as pigment epithelium-derived factor (PEDF; Dawson *et al*., 1999) have also been shown to regulate retinal neovascularization in ischemic retinopathies in patients and animal models.

3.3. Pro-angiogenic factors and their receptors

Oncogene and hypoxia-driven expression of pro-angiogenic factors, such as VEGF, bFGF, Interleukin-8 (IL-8), PlGF, TGF-β and PDGF are one of the key elements in the induction of the "angiogenic switch" (Hanahan and Folkman, 1996).

Of the numerous growth factors contributing to angiogenesis, members of VEGF family of secreted glycoproteins are considered to be key angiogenic mediators contributing to both physiological and pathological angiogenesis. The importance of VEGF for blood vessel formation is demonstrated by the fact that mice with only a single allele of normal VEGF are embryologically lethal (Carmeliet *et al*., 1996; Ferrara *et al*., 1996), as are the mice deficient with VEGFR-1, -2 and –3 (Fong *et al*., 1995; Shalaby *et al*., 1995; Dumont *et al*., 1998). VEGF was first isolated from tumor cells as a factor inducing vascular permeability (Senger *et al*., 1983), and subsequently it has been shown to induce migration, proliferation and survival of endothelial cells and angiogenesis (Ferrara, 1999). The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, the viral VEGF homologue VEGF-E and PlGF. Four different isoforms of VEGF have been identified in human; $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ arising from alternative splicing of VEGF mRNA. The longer forms of VEGF are matrix bound through their heparin-binding domains, while the shorter forms ($VEGF_{121}$ and some of $VEGF_{165}$) are freely diffusible. The longer forms can be released from the ECM by plasmin cleavage, thus increasing local VEGF concentration during tissue growth and remodeling involving proteolysis (reviewed in Veikkola and Alitalo, 1999). Even after the embryonic development, VEGF-A continues playing a crucial role in the formation of new blood vessels; withdrawal of VEGF-A leads to endothelial cell apoptosis and the inhibition of new blood vessel formation resulting in blood vessel regression (Gerber *et al*., 1999). After the newly formed blood vessels have recruited a pericyte coverage, they are no longer dependent on VEGF-A for their survival (Benjamin *et al*., 1998). Whereas VEGF-A is involved in vascular permeability and angiogenesis, VEGF-B is studied for its role in the regulation of ECM degeneration, cell adhesion and migration and VEGF-C and VEGF-D are studied intensively for their role in angiogenesis and lymphangiogenesis in cancer (reviewed in Veikkola and Alitalo, 1999). PlGF-1 induces increased vascular permeability, proliferation, chemotaxis and angiogenesis and may act synergistically with VEGF-A in pathological angiogenesis (Carmeliet *et al*., 2001). Interestingly, if PlGF-1 is produced by the same cells that produce VEGF they form functionally inactive heterodimers that fail to activate VEGFR-2 and VEGFR-2 signaling pathways. High levels of PlGF-1 expression by VEGF-producing tumors force VEGF to dimerize with PlGF-1 resulting in inhibition of angiogenesis and tumor growth (Eriksson *et al*., 2002).

Three VEGF receptor tyrosine kinases have been described; VEGFR-1, -2 and –3. VEGFR-1 binds VEGF-A, VEGF-B and PlGF, VEGFR-2 binds VEGF-A, VEGF-C, VEGF-D and VEGF-E, and VEGFR-3 binds VEGF-C and VEGF-D. NRP-1 binds PlGF-2 and a specific isoform of VEGF-A; $VEGF_{165}$ enhancing the binding of VEGF-A to VEGFR-2 (reviewed in Veikkola and Alitalo, 1999). VEGFR-2 is considered to be the receptor mediating functional VEGF signaling in endothelial cells and there is recent evidence that VEGFR-1 might be predominantly expressed in pericytes in adult human tissues (Witmer *et al*., 2002) mediating the VEGF receptor tyrosine kinase signaling in these cells. VEGFR-3 was initially been reported in lymphatic endothelia and some high endothelial cell venules in adult tissues (Kukk *et al*., 1996), but some studies also describe the expression of VEGFR-3 in quiescent blood vessel endothelium adjacent to epithelium suggesting that VEGFR-3 and its ligands are involved in paracrine communication between endothelial and epithelial cells (Witmer *et al*., 2002). Overexpression of VEGF-C, a VEGFR-3 ligand, results in lymphatic vessel hyperplasia in mice (Jeltsch *et al*., 1997). A critical role for VEGFR-3 signaling in lymphangiogenesis was established in studies with patients with familiar lymphoedema (Karkkainen *et al*., 2000), and the critical role for VEGF-C as the paracrine factor required for the lymphangiogenesis was demonstrated recently (Karkkainen *et al*., 2004). VEGFR-3 has been also found to be involved in angiogenesis in various tumors as well as in wound healing (Paavonen *et al*., 2000; Saaristo *et al*., 2000).

Hypoxia-driven up-regulation of VEGF is the major determinant in the well-characterized model of hypoxia-induced retinal neovascularization (Alon *et al*., 1995; Pierce *et al*., 1995; Stone *et al*., 1995; Okamoto *et al*., 1997). Over-expression of VEGF in the retina is sufficient to cause intraretinal and subretinal neovascularization (Okamoto *et al*., 1997), whereas inhibition of VEGF expression or activity inhibits retinal neovascularization (Aiello *et al*., 1995). VEGF is produced by glial cells of the neural retina, such as specialized astrocytes, including Müller cells among other cell types (Alon *et al*., 1995; Pierce

et al., 1995; Stone *et al*., 1995). VEGF expression in the retina decreases within 6 hours of exposure to 75% oxygen and remains decreased for the duration of the hyperoxia. In contrast, an increase in retinal VEGF expression is observed between 6 and 12 hours after the return to room air, and such expression remains elevated during development of the neovascularization. Therefore, VEGF levels appear to play a dual role in this retinopathy model: a down-regulation of VEGF by hyperoxia induces blood vessel regression, while subsequent up-regulation of VEGF leads to retinal neovascularization (Alon *et al*., 1995; Pierce *et al*., 1996).

The FGFs represent one of the largest families of growth and differentiation factors for cells from mesodermal and neuroectodermal origin. FGF was first purified as a heparin-binding tumor-derived protein stimulating the proliferation of capillary endothelial cells (Shing *et al*., 1984). More than 20 FGFs with different effects on various target cells have been identified and four FGF receptor (FGFR) types have been isolated to date. Two prototypic members of the FGF family, bFGF and acidic fibroblast growth factor (aFGF), are ubiquitously expressed growth factors inducing endothelial cell proliferation, migration and capillary tube formation (reviewed in Risau, 1997 and Ornitz and Itoh, 2001). Fibroblast growth factors stimulate endothelial cell growth and recruit mesenchymal and/or inflammatory cells, producing a variety of angiogenic factors (Carmeliet, 2000). bFGF might not have a general role in angiogenic responses, but it may be necessary for blood vessel remodeling after associated with tissue repair. aFGF and bFGF are not secreted proteins, most FGFs remaining cytoplasmic or binding to the ECM because of their affinity to heparin. FGFs may be released upon a cell injury and they might have a role in angiogenesis following a tissue injury where FGFs are deposited in the ECM (Healy and Herman, 1992). *In vitro* FGFs demonstrate striking synergistic effects with VEGF in the induction of endothelial cell proliferation, migration and capillary tube formation (Goto *et al*., 1993).

TGF-β-1, -2 and -3 are structurally and functionally closely related secreted proteins belonging to a superfamily of multifunctional cytokines. TGF-β isoforms inhibit the proliferation of most cell types, but can act as a growth stimulator of cells from mesenchymal origin, including endothelial cells. TGF-β family members mediate vascular development and regulate a vast number of cellular functions from proliferation, migration, ECM production and deposition to cell differentiation (reviewed in Pepper, 1997). In tumorigenesis, TGF-β plays a dual role in the growth inhibitory pathway downstream of TGF-β, and in the process of epithelial to mesenchymal transdifferentiation (reviewed in Moustakas *et al*., 2002).

In vascular endothelium, TGF-βs are produced as latent forms where TGF-β dimer is bound to latency-associated peptide. On activation from the latent TGF-β complex, TGF-β binds to its constitutively active serine threonine kinase type II receptor (TβR-II) and subsequently forming heterodimers with type I receptors, or activin receptor-like kinases (ALKs 1-7) activating them. ALKs then phosphorylate cytoplasmic receptor-regulated Smads and form complexes with common partner Smads and translocate to the nucleus where they act as sequence specific transcriptional activators. The specificity of TGF-β signaling and subsequent changes in cell function are specified by the expression pattern of type I and type III receptors. In addition to widely expressed TβRII, TβRIII and ALK-5, endothelial cells express ALK-1 type I receptor and endoglin, type IIII receptors phosphorylating Smads 1, 5 and 8 (reviewed in van den Driesche *et al*., 2003). Several ECM proteins can bind to TGF-β and modulate its activity. For example, ECM proteoglycan decorin binds to TGF-β inhibiting its activity while TGF-β binding to TSP-1 leads to increased activity (Yamaguchi *et al*., 1990; Ribeiro *et al*., 1999).

Exogenous TGF-β has been described to have both stimulatory and inhibitory effects on endothelial cells depending on *in vitro* or *in vivo* assays used (reviewed in Pepper, 1997). Studies done using gene knockout mice, have revealed a clear involvement of TGF-β family signaling during vascular development and the maintenance of vascular integrity. TGF-β null mice show defects in endothelial cell differentiation resulting in inadequate capillary tube formation and fragile vessels with reduced cellular adhesiveness (Dickson *et al*., 1995). TGF-β receptor I and II deficient mice also show a similar phenotype (Oshima *et al*., 1996; Larsson *et al*., 2001). These mice show deficiencies in vasculogenesis, in contrast to mice deficient of endoglin, which show no defects in vasculogenesis. Instead, endoglin deficient mice show a failure of endothelial cell remodeling as well as failure to properly recruit mesenchymal cells to growing blood vessels and induce their differentiation to pericytes and vascular smooth muscle cells (Li *et al*., 1999).

From the CXC chemokine family members, IL-8 has long been recognized to function in regulation of pathological angiogenesis, tumor growth and metastasis. (Koch *et al*., 1992; Strieter *et al*., 1995). IL-8 receptors CXCR1 and CXCR2, as well as CXCR4, another receptor for an angiogenic chemokine CXCL12, are expressed on endothelial cell surface (Li *et al*., 2003; Salcedo and Oppenheim, 2003) IL-8 has been shown to directly induce endothelial cell proliferation, survival and capillary tube formation. IL-8 also upregulates endothelial cell expression of MMP-2 and MMP-9 (Li *et al*., 2003). Growth factors can also influence chemokine functions on angiogenic blood vessels. CXCL12 upregulates VEGF-A expression and VEGF-A upregulates CXCR4 expression, thus creating an amplification loop that is influenced by hypoxia-induced growth factor expression (reviewed in Romagnani *et al*., 2004). CXC chemokines provide a fine and

coordinated regulation of angiogenesis and inflammation. The coordination of angiogenesis and inflammation is a result of the ability shared by endothelial cells and leukocytes to respond to chemokines. Interplay between growth factors, cytokines and adhesion molecules regulates the inflammatory response leading to angiogenesis, tissue repair and generation of new tissue (reviewed in Romagnani *et al*., 2004).

3.4. Endogenous angiogenesis inhibitors

There are two classes of angiogenesis inhibitors, direct and indirect. Direct angiogenesis inhibitors prevent endothelial cells from responding to various endothelial cell mitogens or motogens by proliferation, migration or escape from apoptosis. Direct endogenous inhibitors of angiogenesis include angiostatin, endostatin, tumstatin, thrombospondins, vasostatin and PEDF discussed briefly below. Indirect anti-angiogenic factors target proteins that are expressed on tumor cells or their receptors in endothelial cells that are normally activated in angiogenesis (reviewed in Kerbel and Folkman, 2002).

Angiostatin is a 38 kDa internal protein fragment of plasminogen. It contains the first kringle domain of plasminogen and it is produced by the degradation of plasminogen by several MMPs such as MMP-2, MMP-7, MMP-9 and MMP-12. Angiostatin has been shown to have multiple anti-angiogenic activities *in vivo* and *in vitro*. Angiostatin causes regression of tumors, makes endothelial cells resistant to angiogenic stimuli, and induces dormancy of metastasis defined by a balance of proliferating and apoptotic tumor cells (O'Reilly *et al*., 1996; O'Reilly *et al*., 1994, reviewed in O'Reilly, 2002). Additionally, angiostatin induces apoptosis of endothelial cells (Claesson-Welsh *et al*., 1998). Angiostatin has been shown to bind ATP synthase, angiomotin and annexin II on endothelial cells (reviewed in Kerbel and Folkman, 2002).

Two angiogenesis inhibitors derive from precursors of collagen molecules. Endostatin is a 20 kDa proteolytic fragment of carboxy-terminal collagen XVIII and tumstatin is a fragment of type IV collagen. Endostatin is an endogenous and specific inhibitor of endothelial cell proliferation, migration, invasion, capillary tube formation and angiogenesis (O'Reilly *et al*., 1997). Several proteases have been described to cleave collagen XVIII producing endostatin, cathepsin L being the most potent one. Other proteases include cathepsin B, elastase and several MMPs (Felbor *et al*., 2000; Ferreras *et al*., 2000). The first identified mechanisms for endostatin effects on endothelial survival were inhibition of the two anti-apoptotic proteins Bcl-2 and Bcl-XL (Dhanabal *et al*., 1999). Inhibitory action by endostatin has been proposed to involve binding to $\alpha_5\beta_1$ integrin leading to the inhibition of focal adhesion kinase/c-Raf/MEK1/2/p38/ERK1 mitogen-activated protein kinase pathway (Sudhakar *et al*., 2003). More recently, endostatin was shown to downregulate many signaling pathways associated with pro-angiogenic activity on microvascular endothelial cell, and upregulate many anti-angiogenic genes. For instance, signaling pathways associated with TSP-1 regulation have been shown to be tied to endostatin. Endostatin upregulates TSP-1 while suppressing Id—1, a downregulator of TSP-1 (Abdollahi *et al*., 2004).

Tumstatin, a 28 kDa fragment of type IV collagen binds to $\alpha_v\beta_3$ integrin, inhibits the proliferation of endothelial cells *in vitro* and angiogenesis and tumor growth *in vivo* selectively stimulating apoptosis of endothelial cells (Maeshima *et al*., 2000a; Maeshima *et al*., 2000b). Tumstatin has been shown to function as an endothelial cell specific inhibitor of cap-dependent protein synthesis. Through interaction with $\alpha_v\beta_3$ integrin, tumstatin inhibits the activation of focal adhesion kinase, PI3K, protein kinase B and mTOR, the mammalian target of rapamycin, and prevents the dissociation of eIF4E, eukaryotic initiation factor 4E protein from 4E-binding protein 1 (Maeshima *et al*., 2002).

Thrombospondin is an ECM molecule and it is also an inhibitor of endothelial cell adhesion, motility, proliferation and angiogenesis *in vivo* (Good *et al*., 1990). It is a protein consisting of three identical disulfide-linked 180kDa chains and exists in two isoforms: TSP-1 and TSP-2. CD36 is the cellular receptor for TSP-1 on microvascular endothelial cells and it is necessary for the anti-angiogenic activity of TSP-1. A histidine-rich glycoprotein that is present in high concentration in the plasma binds to TSP-1 interfering with the interaction of TSP-1 with CD36 thus opposing the anti-angiogenic effect of TSP-1 (Dawson *et al*., 1997). TSP-2 is highly expressed in developing vessels. Disruption of the thrombospondin 2 gene (*Thbs2*) in mice results in a complex phenotype characterized mainly by abnormalities in fibroblasts, connective tissues, and blood vessels. Disruption of *Thbs2* is characterized by increased angiogenesis during foreign body reaction (Kyriakides *et al*., 1999). TSP-2 has been shown to exert a strong anti-angiogenic effect on squamous cell carcinomas in mice by significantly reducing the density and size of intratumoral vessels. Combined expression of TSP-1 and TSP-2 completely suppress squamous cell carcinoma development, suggesting that a combination of these angiogenesis inhibitors may provide better therapeutic efficiency than TSP-1 or TSP-2 alone (Streit *et al*., 1999). TSP-1 has been demonstrated to be one of the key regulators of the angiogenic switch in a genetically defined tumor model. In this model, cooperative activity of the oncogenes, ras and myc, Ras inducing the sequential activation of PI3K, Rho, and ROCK, leading to activation of Myc, leads directly to angiogenesis and tumor formation by repressing TSP-1 expression (Watnick *et al*., 2003).

Vasostatin is a molecule composed of 180 amino acid from the N-terminal domain of the human calreticulin. It is a potent and selective inhibitor of endothelial cell proliferation, and it has anti-

angiogenic activity *in vivo* (Pike *et al*., 1998). Vasostatin was shown to selectively affect tumor angiogenesis, but not physiological angiogenesis during wound healing (Lange-Asschenfeldt *et al*., 2001).

Some chemokines have anti-angiogenic activity. CXCL4 and CXCL10 inhibit angiogenesis by interfering with the binding of bFGF or $VEGF_{165}$ to their receptors or by several receptor-dependent mechanisms (reviewed in Romagnani *et al*., 2004) Pigment-epithelium-derived factor (PEDF) that is expressed in the retina, is a potent inhibitor of angiogenesis and is one of the key regulators in maintaining vitreous and cornea of the eye avascular. PEDF was shown to inhibit neovascularization of the cornea in a rat model *in vivo*, as well as endothelial cell proliferation and migration *in vitro* (Dawson *et al*., 1999). Unlike other angiogenesis inhibitors, PEDF is directly regulated by hypoxia at the translational or post-translational level, hypoxia decreasing PEDF levels and hyperoxia increasing them (Dawson *et al*., 1999). The anti-angiogenic activity of PEDF is not limited to the retinal microenvironment. Overexpression of PEDF in melanoma cells was demonstrated to inhibit tumor growth by two different mechanisms; by inhibition of tumor angiogenesis and by induction of Fas ligand-dependent apoptosis in tumor cells (Abe *et al*., 2004).

Interferon (IFN)-α and IFN-β are indirect endogenous inhibitors of angiogenesis (Kerbel and Folkman, 2002). IFN-α and IFN-β downregulates the tumor cell expression of bFGF by a mechanism independent of their antiproliferative effects (Singh *et al*., 1995), thus leading to diminished angiogenic signals for endothelial cells.

3.5. Role of proteases and peptidases in blood vessel formation

Degradation of ECM proteins is essential for blood vessel formation and remodeling during development, growth, and tissue repair. On the other hand, excessive degradation of the ECM has an important role in many pathological conditions including rheumatoid arthritis and cancer (reviewed in Egeblad and Werb, 2002, and Murphy *et al*., 2002). Proteolytic enzymes are classified as either exopeptidases or endopeptidases based on whether they cleave terminal or internal peptide bonds, respectively.

3.5.1. Matrix metalloproteinases

MMPs are a large family of metallo-endopeptidases that degrade the protein components of the ECM and basement membrane. MMPs play important roles in wound healing, angiogenesis, embryogenesis and in pathological processes such as tumor invasion and metastasis, basically in every process involving tissue remodeling. Historically MMPs have been divided into four groups; collagenases, gelatinases, stromeolysins and matrilysins, based on their ECM substrate specificity. Nowadays they are divided into different structural groups from which five are secreted and three membrane-type MMPs (MT-MMPs). MT-MMPs are covalently linked to the cell membrane, but even the secreted MMPs can be associated with the cell membrane by binding to cell surface molecules such as integrins, CD44 or cell surface-associated heparan sulphate proteoglycans, collagen type IV and EMMPRIN co-localizing their proteolytic activity to site of cell-ECM contact (reviewed in Egeblad and Werb, 2002, and Stamenkovic, 2003).

MMP activity is controlled by the activation of their transcription, proteolytic activation of the zymogen form (all MMPs are produced as zymogens; they contain a pro-peptide whose cleavage is required for MMP activation) and by the inhibitory activity of a variety of endogenous MMP inhibitors (reviewed in Egeblad and Werb, 2002, and Stamenkovic, 2003). Most MMPs can be activated by the proteolytic activity of other MMPs and a variety of serine proteases. α2-macroglobulin is the major MMP inhibitor in tissue fluids leading to the clearance of MMP-α2-macroglobulin complex by scavenger receptors (Sottrup-Jensen and Birkedal-Hansen, 1989). Other MMP-inhibitors include TIMPs which bind reversibly to MMPs blocking enzyme activity (Gomez *et al*., 1997). TSP-1 binds to proMMP-2 and -9 blocking their activation while TSP-2 mediates MMP-2 clearance by scavenger receptor (Bein and Simons, 2000; Rodriguez-Manzaneque *et al*., 2001). The only cell surface MMP inhibitor known is RECK, which has been shown to inhibit MMP-2, MMP-9 and MT1-MMP (Oh *et al*., 2001).

Degradation of ECM substrates does more than create space for growing and migrating cells. Since cells have receptors, like integrins, for structural ECM components, degradation of ECM components affects cell signaling trough these receptors. Cleavage of laminin-5 and type IV collagen results in exposure of pro-migratory cryptic sites. Cleavage of insulin-like growth factor-binding protein and perlecan releases insulin-like growth factor. MMP-2 and MMP-9, when localized on the cell membrane, can cleave the latent form of TGF-β leading to its activation (Yu and Stamenkovic, 2000). Another example is the cleavage of SPARC, a matricellular protein of the ECM, by MMP-3. Produced peptides can regulate endothelial cell proliferation and/or migration and angiogenesis (Sage *et al*., 2003). In addition to the proteolytic degradation of ECM molecules, recent data has extended the substrate specificity of MMPs to include enzyme inhibitors, cell-bound precursors of cytokines and active cytokines, cell adhesion molecules and growth factor receptors. MMPs participate in releasing cell-bound growth factor receptor precursor forms, including TGF-α. Some growth factor receptors are also substrates for MMPs. Different MMPs cleave FGFR-1, ERBB2, ERBB4 and hepatocyte growth factor receptor c-MET resulting in formation of soluble decoy-receptors. Cleavage of cell adhesion molecules E-cadherin,

CD44 and the α_v-integrin enhances tumor cell invasion and migration (reviewed in Egeblad and Werb, 2002, and Stamenkovic, 2003).

MMPs are upregulated in majority of human cancers, and their expression is often associated with poor survival. In addition to the role MMPs play in the formation of the tumor vasculature, they can promote cancer progression by increasing the growth rate, invasion, migration and metastasis potential of tumor cells (reviewed in Egeblad and Werb, 2002).

3.5.2. Aminopeptidases

Aminopeptidases are a large group of enzymes widely encountered in nature. They are involved in a number of biological processes such as maturation, regulation and degradation of proteins and polypeptides. The expression and enzymatic activity of some of the aminopeptidases have been shown to play a role in the angiogenesis and tumor growth. These aminopeptidases include methionine aminopeptidase-2 (MetAP-2), aminopeptidase N (APN) and aminopeptidase A (APA).

Methionine aminopeptidases (MetAP-1 and MetAP-2) are monomeric cobalt metallopeptidases that remove the initial N-terminal methionine residues from polypeptides. MetAP-2 is a ubiquitous cytosolic housekeeping enzyme. It was found to be a target of a potent angiogenesis inhibitor fumagillin analogue TNP-470, which binds covalently to MetAP-2 inhibiting its enzymatic activity (Ingber *et al.*, 1990; Sin *et al.*, 1997). Since the inhibition of the MetAP-2 inhibits the proliferation of endothelial cells *in vitro* and *in vivo* mediating the anti-angiogenic effect of TNP-470, there has to be a level of targeting the effect of the drug to the proliferating endothelial cells. This level of targeting comes most likely from the specific MetAP-2 substrates that do exist in the proliferating endothelial cells. MetAP-2 is responsible for removing the methionine from specific proteins in endothelial cells and inhibition of this process affects a wide range of post-translational modifications controlling protein activation, translocation and turnover (reviewed in Bradshaw and Yi, 2002).

Cloning of CD13 revealed that this protein is identical to APN (Look *et al.*, 1989). CD13/APN (E.C. 3.4.11.2) is a highly conserved 150 kDa transmembrane glycoprotein, which is incorporated into the cell membrane through its N-terminal hydrophobic segment (Look *et al.*, 1989; Xu *et al.*, 1997). The large, extracellular carboxy- terminal domain contains a pentapeptide (HEXXH-motif, HELAH in CD13/APN) characteristic of many zinc-dependent metalloproteases (Look *et al.*, 1989). APN has zinc metallopeptidase activity hydrolyzing unsubstituted, N-terminal amino acids with neutral side chains (Ala>Phe>Leu>Gly) from oligopeptide substrates (Lalu *et al.*, 1986; Roques *et al.*, 1993).

CD13/APN was originally thought to be a marker restricted to subsets of normal and malignant myeloid lineage cells (Drexler, 1987; Favaloro *et al.*, 1988; Amoscato *et al.*, 1989; Ashmun and Look, 1990; Makrynikola *et al.*, 1995). It is now known to be expressed in many different cell types (Look *et al.*, 1989), including vascular endothelium, smooth muscle cells (Palmieri *et al.*, 1985) and pericytes (Kunz *et al.*, 1994; Alliot *et al.*, 1999), antigen presenting cells (mainly B cells, macrophages, dendritic cells, and veiled cells; (Hansen *et al.*, 1993) and keratinocytes (Gabrilovac *et al.*, 2004), as well as in established tumor cell lines (Amoscato *et al.*, 1990) and in many tumor tissues, including pancreatic carcinoma (Ikeda *et al.*, 2003), prostate cancer (Ishii *et al.*, 2001), mesenchymal tumors (Mechtersheimer and Moller, 1990), melanomas (Menrad *et al.*, 1993) and clear cell renal carcinomas (Nanus *et al.*, 1998). The *CD13/APN* gene has two different promoters. The proximal promoter directs *CD13/APN* transcriptional regulation in kidney, intestine, and liver epithelial cells, whereas the distal promoter controls *CD13/APN* expression in myeloid cells and fibroblasts. The transcripts from both promoters encode the same CD13/APN protein as they differ only in their 5' untranslated regions (Shapiro *et al.*, 1991).

Many natural substrates have been discovered for APN; vasoactive peptides lysyl-bradykinin and angiotensin III (AngIII), neuropeptide hormones leu/met-enkephalin, neurokinin A, and somatostatin, and some cytokines and immunomodulatory peptides, like tuftsin and IL-8 (reviewed in Riemann *et al.*, 1999). In synaptic membranes CD13/APN metabolizes enkephalins and endorphins (Matsas *et al.*, 1984; Konkoy *et al.*, 1996) and in the intestinal brush border, it degrades regulatory peptides and scavenges amino acids (Turner *et al.*, 1987; Rawlings and Barrett, 1993); in lymphocytes, its activity is associated with mitotic activation, antigen processing (Mouritsen *et al.*, 1992; Falk *et al.*, 1994), cell adhesion, and migration (Koch *et al.*, 1991; Menrad *et al.*, 1993; Saiki *et al.*, 1993). CD13/APN has also been implicated in tumor invasion (Saiki *et al.*, 1993; Fujii *et al.*, 1995; Ishii *et al.*, 2001), signal transduction in myeloid cells (Lohn *et al.*, 1997; Santos *et al.*, 2000), cell cycle control and differentiation (Riemann *et al.*, 1997; Lohn *et al.*, 2002). CD13/APN has also been suggested to have a role as a scavenger of short, nonspecific peptides in the circulation (Janas *et al.*, 2002). Lymphocytic expression of CD13 represents a potentially increased cellular ability to inactivate inflammatory mediators. Furthermore, CD13/APN could be involved in adhesion, in lymphocytic migration, or in the antigen processing of peptides bound in the groove of MHC class II molecules (Makrynikola *et al.*, 1995; Riemann *et al.*, 1997), and as a receptor for corona viruses (Delmas *et al.*, 1992; Yeager *et al.*, 1992). Several factors have been shown to induce the expression of CD13/APN. A direct cell-cell contact of lymphocytes with CD13/APN expressing adherent cells, such as

fibroblast-like synoviocytes, human umbilical vein endothelial cells (HUVECs), epithelial cells and macrophages, induces CD13/APN expression on lymphocytes (Riemann *et al.*, 1997). IL-4 stimulation increases CD13/APN activity in human monocytes, macrophages, and endothelial cells (van Hal *et al.*, 1994) as do some other T-cell derived cytokines, such as interferon-γ and IL-13 (Riemann *et al.*, 1995). Studies using monoclonal antibodies indicate that CD13/APN undergoes regulatory intramolecular alterations that result in exposure of cryptic sites and regulation of enzyme activity (Xu *et al.*, 1997). The presence of certain epitopes has also been related to prognosis of acute myeloid leukemia (Favaloro *et al.*, 1988; Makrynikola *et al.*, 1995; Xu *et al.*, 1997).

Cell-surface CD13/APN enzymatic activity can be blocked by bestatin and o-phenatroline (Rawlings and Barrett, 1993; Saiki *et al.*, 1993; Taylor, 1993). Studies involving these inhibitors have contributed to our understanding of the enzyme function. For example, bestatin has been shown to possess immunomodulatory effects (Bruley-Rosset *et al.*, 1979; van Hal *et al.*, 1994). It has also been reported that administration of high doses of bestatin suppresses experimental or spontaneous metastasis and inhibits tumor cell invasion, apparently by inhibiting the degradation of the ECM, particularly collage type IV (Yoneda *et al.*, 1992; Saiki *et al.*, 1993; Fujii *et al.*, 1995). However, specific targets for CD13/APN in the context of tumor progression remain unknown.

APA (glutamyl-aminopeptidase, EC 3.4.11.7) is a homodimeric type II membrane-spanning cell surface protein consisting of a short N-terminal cytoplasmic domain, a single transmembrane domain and a large extracellular domain containing the active site (including the HEXXH-motif, HELVH in APA; Wang *et al.*, 1996) with zinc metallopeptidase activity that hydrolyzes N-terminal glutamyl or aspartyl residues from oligopeptide substrates (Danielsen *et al.*, 1980; Lalu *et al.*, 1986; Yamada *et al.*, 1988). APA shares significant sequence homology with CD13/APN (Malfroy *et al.*, 1989; Wu *et al.*, 1990; Nanus *et al.*, 1993).

The murine BP-1 antigen/APA was originally identified as a cell surface glycoprotein expressed by pre-B and immature B cells (Cooper *et al.*, 1986), and it was shown to be identical to 6C3 antigen on leukemic pre-B cells (Wu *et al.*, 1989). Human APA was first cloned be a human kidney differentiation antigen gp160 (Nanus *et al.*, 1993) or 140-160 kDa type II membrane bound metallopeptidase identical to the mouse B cell differentiation antigen (Li *et al.*, 1993a). The restricted expression of APA during B cell differentiation (Cooper *et al.*, 1986), and its elevated expression in response to IL-7 (Wang *et al.*, 1996) suggest a role for APA in the developing immune system, but studies with APA deficient mice suggests, that besides mice developing normally, they also exhibit normal B and T cell development (Lin *et al.*, 1998). APA has also been suggested to have a role in the central nervous system in degrading cholecystokinin-8 peptide (Migaud *et al.*, 1996). The only well understood role for APA is the conversion of AngII to AngIII in the renin-angiotensin system (Jackson, 2001). AngII, a key element in the renin-angiotensin system regulating blood pressure is in addition to cholecystokinin-8 (Migaud *et al.*, 1996) the only natural substrate characterized for APA to date (Nagatsu *et al.*, 1970). APA as an enzyme responsible for the formation of AngIII represents a potential therapeutic target for antihypertensive agents, since AngIII is the major effector peptide of the renin angiotensin system at least in the brain, exerting tonic stimulatory control over blood pressure (Reaux *et al.*, 1999). These findings were supported by studies in APA deficient mice that were found to have elevated baseline blood pressure. Furthermore, exogenous AngII lead to an enhanced hypertensive response in these mice indicating a physiological role for APA in lowering blood pressure (Mitsui *et al.*, 2003). APA is expressed in placental trophoblasts and it appears to have an important role in the maintenance of homeostasis during pregnancy by regulating trophoblast function via degradation of AngII (Hariyama *et al.*, 2000; Mizutani *et al.*, 1981) acting as a barrier to AngII between mother and the fetus.

APA has been shown to have a widespread tissue distribution including the small intestine intestinal brush border, bile canaliculi in liver, proximal renal tubules and renal glomeruli, pulmonary alveolar walls and blood vessels of many organs in murine tissues using both enzyme histochemistry (Lojda and Gossrau, 1980) and immunohistochemistry (Li *et al.*, 1993b). The blood vessel staining has been interpreted as endothelial cell reactivity, but at least in rat and mouse brain blood vessels, the APA expression has been demonstrated to be solely on pericytes (Healy and Wilk, 1993; Alliot *et al.*, 1999), suggesting that pericytes might be the source of microvascular APA in other organs as well. APA is also known to be expressed in variety of tumor cell types including neoplastic cervical epithelial cells (Fujimura *et al.*, 2000), neoplastic trophoblasts in choriocarcinomas (Ino *et al.*, 2000) and clear cell renal carcinomas (Nanus *et al.*, 1998).

Activated pericytes have been described to show a marked expression of APA in angiogenic vasculature. Microvessels of human tumors and granulation tissue show marked expression of APA recognized by the monoclonal antibody RC38 (Oosterwijk *et al.*, 1986), whereas the expression of APA on blood vessels of normal organs is weak or absent. APA expression in the tumor vasculature was shown to be restricted to the activated pericytes and their processes (Schlingemann *et al.*, 1996). Interestingly, these studies were done by using the RC38 antibody, that recognize a subspecies of APA (most likely underglycosylated; Oosterwijk *et al.*, 1986) indicating that different cryptic epitopes of APA or different forms of the enzyme can be exposed

in a specific site depending on the tissue microenvironment (Schlingemann *et al*., 1996).

4. Blood vessel heterogeneity

The organ-, tissue- and vessel-specific differences in the vasculature associated with functional differences in normal blood vessels, and environmental differences of blood vessels in diseased tissues contribute to the concept of the heterogeneity of the vasculature.

Endothelial cells covering the inner surfaces of blood vessels are heterogeneous both in structure and at the level of expression patterns of cell surface molecules. Endothelial cells vary in size and shape, thickness, in the orientation of the nucleus, in the presence of microvilli and plasmalemmal vesicles. Endothelial cells can also have a variety of different kinds of cell junctions, contributing to the differential barrier functions at different sites of endothelium. Heterogeneity of endothelial cells at the expression patter level is reflected by the differences in the cell surface glycoprotein and lectin binding patterns and mRNA and protein expression (reviewed in Aird, 2003). All these structural and molecular differences are the primary determinants of the differences in blood vessel function.

4.1. Heterogeneity of the normal vasculature

4.1.1 Structural diversity of the normal vasculature

Electron microscopic studies in the 50s examining blood vessels in different organs lead to a structural classification of capillaries into three general categories; continuous, discontinuous and fenestrated capillaries (Bennett *et al*., 1959). Capillaries in skeletal muscle, heart, lung and brain have a continuous endothelium while capillaries in the liver, spleen and bone marrow have a discontinuous endothelium that allows cell trafficking between intracellular gaps. Capillaries in many endocrine and exocrine glands, choroid plexus, intestinal villi and gall bladder have endothelial fenestrations covered by diaphragms while capillaries of renal glomeruli have endothelial fenestrations without the covering diaphragm. (Simionescu and Simionescu, 1984).

Blood vessels of the brain and the retina are examples of extremely specialized continuous endothelium. Endothelial cells in the brain and the retina are distinguishable from endothelial cell of non-central nervous system origin by their "barrier" status; blood-brain barrier in the brain and blood-retinal barrier in the retinal vasculature. In addition to endothelial cells and pericytes, astrocytes and microglia are important structural components of the retinal and brain blood vessels contributing to the perivascular *glia limitans* separating the circulation from the central nervous system parenchyma (reviewed in Provis, 2001).

The mature phenotype of quiescent organ vasculature in most organs is characterized by an extensive coverage with pericytes that appear to play a role in maintaining the quiescent phenotype of the endothelium (Morikawa *et al*., 2002).

4.1.2. Molecular diversity of the normal vasculature

Endothelial cells express receptors on their surface for a variety of ligands such as proteins, hormones, metabolites and lipid-transporting particles. They also express a wide array of cell-cell junction proteins and adhesion proteins for cell-cell and cell-ECM interactions. Genetic predisposition and the influence of the microenvironment are the main determinants of the molecular heterogeneity between endothelial cells. Organ-specific phenotypes are either predetermined before the endothelial cell precursors migrate from the mesoderm to a specific vascular bed or local cues from the vascular bed microenvironment determine the phenotype of the endothelial cell.

First isolated differentially expressed molecules on vascular endothelium were involved in the homing of leukocytes to particular organs. High endothelial venules are specialized postcapillary venules in lymphoid tissues that support high levels of lymphocyte extravasation from the blood (Bevilacqua, 1993). High endothelial venules differ from the vascular postcapillary venules in their structural and molecular composition, but they also show tissue specific differences. For example mucosal addressin cell adhesion molecule-1 is selectively expressed on endothelium of high endothelial venules in gut and gut-associated lymphoid tissue. By interaction with its integrin ligand, $\alpha_4\beta_7$, lymphocytes presumed to be involved in mucosal immunity are selectively recruited to these intestinal sites (Briskin *et al*., 1997).

Tumor cells can also show preferential adhesion to the endothelium of specific organs paralleling their metastatic propensities (McCarthy *et al*., 1991).

It has been established, that differences between arteries and veins are in part genetically determined. Arterial and venous endothelial cells are molecularly distinct from the earliest steps of angiogenesis during embryogenesis. Even before the onset of circulation, endothelial cells destined to line the arteries express ephrin-B2 and endothelial cells fated to line the veins express Eph-B4, a receptor for ephrinB2 (Wang *et al*., 1998) thus helping to define the arterial-venous identity. The normal sprouting and branching of the developing vasculature involves signaling preventing the arteries and veins from fusing together. Notch-Gridlock signaling pathway induces the expression of arterial genes and suppression of venous-specific genes controlling the assembly of first embryonic arteries (Lawson *et al*., 2001; Zhong *et al*., 2001). Also microvascular and macrovascular endothelial cells

from the same organ differ in their lymphocyte adhesion receptor expression patterns, their ability to synthesize prostaglandins and to form capillary-like structures (Ades *et al.*, 1992). Ang-1 and Tie1 in combination are critical in establishing vascular polarity during angiogenesis as a part of a distinct genetic program for the establishment of the right-hand side and left-hand side vascular networks well before the network asymmetry becomes morphologically apparent (Loughna and Sato, 2001). Also the decision to form a vein or artery is made early on during embryogenesis, before the start of the circulation (Wang *et al.*, 1998). Whereas endothelial linage is genetically predetermined, expression of a set of vascular bed specific genes can be regulated at a transcriptional level after interaction with local signaling pathways (Aird *et al.*, 1997).

vWF is differentially expressed and regulated in endothelial cells present in different tissues and within the same vascular bed. It is expressed predominantly in the endothelial cells of veins (Yamamoto *et al.*, 1998). Tissue factor inhibitor pathway is a marker of microvascular endothelium (Osterud *et al.*, 1995), while thrombomodulin is expressed in the vasculature of all organs except the brain (Ishii *et al.*, 1986). Section 5.2.1. will review the studies done to map the molecular diversity of the endothelium using *in vivo* phage display technology.
While endothelial cell heterogeneity has been long recognized, the exact molecular mechanisms underlining this heterogeneity remain largely unknown. The molecular diversity of endothelial cells may arise from factors inherent in the cells or signals in the cell microenvironment (Stevens *et al.*, 2001).

Differential glycosylation of endothelial cell surface proteins and proteoglycans may also contribute to the molecular heterogeneity of the vasculature. Immunohistochemical studies of functionally active glycan-decorated L-selectin ligands (sialyl Lewis X) or sulfated extended core 1 lactosamine has revealed upregulation of sialyl Lewis X or sulfo sialyl Lewis X glycans on endothelial cell surface in inflamed tissues (ulcerative colitis, psoriasis, thyroiditis, myocarditis and vasculitis). In contrast, non-inflamed endothelium does not express sialyl Lewis X or sulfo sialyl Lewis X epitopes. Furthermore, the expression pattern of sialyl Lewis X or sulfo sialyl Lewis X epitopes is different between all the analyzed organs suggesting an existence of a glycosylation-based "zip code" of the vasculature (Renkonen *et al.*, 2002).

Caveolae has also surfaced as an interesting new target in the heterogeneity of the vasculature. Caveolae are specialized distinct plasma membrane microdomains and the associated noncoated plasmalemmal vesicles (Palade, 1953) on several cell types, including the cells of the continuous microvascular endothelium. Many proteins have been found enriched in caveolae, including cell surface receptors such as platelet-derived growth factor receptors, epithelial growth factor receptors, basic fibroblast growth factor receptor and endothelin receptors (reviewed in Zajchowski and Robbins, 2002). Even though the molecular differences of caveolae between endothelial cells derived from different tissues remain unknown, recent data suggests that caveolae can contain tissue-specific cell surface molecules. A lung endothelial cell specific antibody targeting the caveolae has been generated using an antibody- and subfractionation strategy. Upon intravenous administration, this anti-lung caveolae –antibody localized to the microvascular endothelium of rat lungs. In addition, targeting caveolae increased trasendothelial transport of the anti-caveolae antibody (McIntosh *et al.*, 2002).

It is becoming evident that endothelial cells in lymphatic vessels are also heterogeneous, both in structure and in protein expression patterns. Endothelial cells of the lymphatic vessels and blood vessels from the same organ show a distinct expression of large number of lineage-specific genes (Hirakawa *et al.*, 2003). Only a small percentage of the profiled genes show differential expression between the endothelial cells derived from blood vessels or lymphatic vessels corroborating the close relationship of the blood vascular and lymphatic vascular system (Hirakawa *et al.*, 2003). Tumor lymphatic vessels have also been suggested to carry specific markers that are undetectable in normal lymphatic vessels (Laakkonen *et al.*, 2002).

4.1.3. Species specific differences

It is possible to use information about protein expression patterns, peptide ligands and their receptors on mouse blood vessels for the development of vascular targeted therapies to treat human diseases. Extrapolation of the results from mouse experiments to human biology, however, requires that the molecules of interest be expressed and regulated similarly in both species. It has recently become apparent that data derived from mouse models are often not easily translated into clinical applications, and results with some mouse data derived anti-cancer drugs have been disappointing (Coussens *et al.*, 2002).

Data from the Human Genome Project indicate that the higher complexity of the human species relative to other mammalian species derives from expression patterns of proteins at different tissue sites, levels or times rather than from a greater number of genes (Lander *et al.*, 2001; Venter *et al.*, 2001). Several examples of cross-species variation of gene expression patterns within the vascular network have recently surfaced. For example, the prostate-specific membrane antigen (PSMA) shows notably different expression pattern in human and mice. PSMA in human is expressed in prostate; in mouse on the other hand, the expression of PSMA is limited to the brain and kidney (Bacich *et al.*, 2001). Additionally, PSMA is an endothelial cell marker of human tumor blood vessels (Chang *et al.*, 1999), whereas

mouse tumor blood vessels do not have a detectable endothelial expression of PSMA (W.D.W. Heston, *personal communication*). Another example of cross-species variation is the *TEM7* gene, which is highly and selectively expressed in the endothelium of human colorectal adenomas (St Croix *et al.*, 2000). In Mouse, mTEM7 is expressed in Purkinje cells of the cerebellum, while the tumor blood vessels show no mTEM7 expression (Carson-Walter *et al.*, 2001). There are also species specific differences in the induction of protein expression by cytokines. For example, tumor necrosis factor-α (TNF-α) and oncostatin M function cooperatively to induce vascular expression of P- and E-selectin in mice but diverge significantly in their effects on expression of P- and E-selectin in humans or nonhuman primates (Yao *et al.*, 1999). Species-specific differences in protein expression and ligand–receptor accessibility should caution us about translating vascular targeting data obtained in animal models directly to human studies.

4. 2. Heterogeneity of the angiogenic vasculature

The rules of the normal blood vessel formation do not seem to apply to tumor blood vessel formation. There is a tremendous amount of functional and structural irregularity and molecular heterogeneity in tumor/angiogenic blood vessels when compared to normal/quiescent blood vessels. The same general ideas that apply to the heterogeneity of the normal, quiescent, vasculature, can be applied to angiogenic blood vessels as well. The angiogenic blood vessels are heterogeneous, both on a structural and on a molecular level; certain abnormalities can develop in some tumor blood vessels and not in others.

4.2.1. Structural diversity of the angiogenic vasculature

Tumor blood vessels have an abnormal morphology. They are irregular in diameter and branching pattern and they do not exhibit the defined structural features of arterioles, capillaries or venules (Less *et al.*, 1991; reviewed in McDonald and Foss, 2000). Tumor blood vessels are not lined with a normal monolayer of endothelial cells, instead the vessel lining is composed of endothelial cells that are disorganized, loosely and irregularly connected, branched, overlapping and sprouting. Many tumor blood vessels also have abluminal endothelial sprouts that penetrate deep into the perivascular tumor tissue. Since endothelial cells do not form a normal monolayer, they do not perform the normal barrier function of the endothelium (Hashizume *et al.*, 2000; McDonald and Foss, 2000).

Proliferating cancer cells can also cause structural changes in tumor blood vessels causing intratumor blood vessels, especially ones without supportive stromal structures, to collapse (Padera *et al.*, 2004).

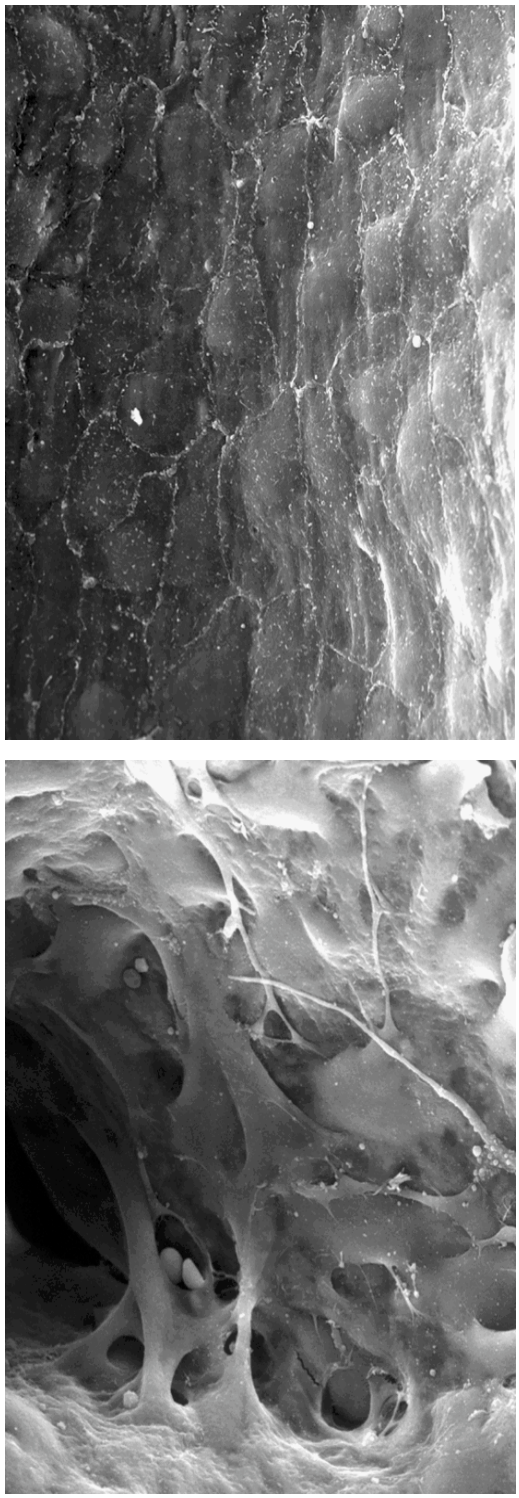

Figure 3. Scanning-electron microscopic images of the luminal surface of blood vessels in normal mouse mammary gland and in mammary carcinoma. In a normal mammary-gland venule (upper panel), endothelial cells form a monolayer on the luminal surface. The cells are relatively uniform in size and shape, flat and tightly apposed to one another. In a tumor blood vessel, endothelial cells on the luminal surface are severely deformed, separated by wide intercellular spaces, overlap one another and have cellular projections. Adapted with permission, from Hashizume *et al.*, 2000.

Most of the studied tumor blood vessels in spontaneous and implanted murine tumor models are covered with pericytes. However, pericytes surrounding the endothelial cells display severe morphological abnormalities. These pericytes are abnormally loosely associated with the endothelial cells with wide spaces separating the two types of cells. They extend cytoplasmic extensions deep into the tumor tissue, unlike in normal blood vessels. The pericyte sleeves surrounding the tumor blood vessels are also significantly longer that the endothelial cell sprouts themselves. The abnormal interactions of pericytes with endothelial cells within tumor blood vessels may alter the influence pericytes have on endothelial cell morphology and function, and may contribute to the leakiness of tumor blood vessels (Morikawa *et al*., 2002).

Confocal microscopic studies have revealed that despite the basement membrane covering the surface of more than 99.9% of the blood vessels in tumors, it exhibits conspicuous structural abnormalities including broad extensions away from the vessel wall and loose associations with endothelial cells and pericytes (Baluk *et al*., 2003).

The influence of tumor microenvironment in tumor development is increasingly appreciated (Liotta and Kohn, 2001). Rather than being renegades, tumor cells recruit vasculature and stroma through the production and secretion of growth factors and cytokines (Brown *et al*., 1999). The locally activated microenvironment (both cellular and the extracellular elements) in turn modifies the proliferative and invasive behavior of tumor cells (Vaccariello *et al*., 1999). As developing neoplasias activate angiogenesis and recruit neovasculature from the surrounding microvascular bed, the tissue microenvironment likely influences the morphology and physiology of the developing tumor vasculature. Comparing the permeability of the vessels in transplanted tumors as a function of tumor type and host tissue site, organ specific differences in tumor blood vessels are starting to emerge (Hobbs *et al*., 1998). Expression and activity of general angiogenic factors, such as VEGF and Ang-1 varies in different tissues. Tumors that have highly permeable blood vessels lack Ang-1, where as tumors with "tight", low-permeability blood vessels overexpress Ang-1 and/or down-regulate VEGF expression (Jain and Munn, 2000).

By characterizing endothelial cell proliferation in six different human tumor types, Eberhard and colleagues (Eberhard *et al*., 2000) found angiogenesis to be present in all of the tumors with a characteristic and significant differences between the tumor types. Notably, even in the most angiogenic tumors, angiogenesis was up to 20-fold less intense than in the physiological angiogenesis (cyclic bovine ovarian corpus luteum). Also the degree of pericyte recruitment to neovasculature in different of tumors as well as within a given tumor type have been shown to vary significantly probably indicating the differences in functionality/maturity of the tumor vascular bed (Eberhard *et al*., 2000).

4.2.2. Molecular diversity of the angiogenic vasculature

Angiogenic vasculature expresses markers that are either expressed at much lower levels or not at all in non-proliferating endothelial cells (St Croix *et al*., 2000, and reviewed in Ruoslahti, 2002 and Trepel *et al*., 2002). Markers of angiogenic blood vessels include receptors for vascular growth factors, such as specific subtypes of VEGF and bFGF receptors (Plate *et al*., 1993; Brekken *et al*., 1998; Valtola *et al*., 1999), α_v-integrins (Brooks *et al*., 1994a; Brooks *et al*., 1994b), proteoglycans (Schrappe *et al*., 1991; Burg *et al*., 1999) and proteinases (Koivunen *et al*., 1999). Several ephrin receptors are also overexpressed in tumors especially during the more invasive stages of tumor progression (reviewed in Dodelet and Pasquale, 2000). Ephrin-A1 ligand and its receptor EphA2 are expressed throughout tumor vasculature in a mouse model of human breast carcinoma and human tumor sections and may play a role in tumor angiogenesis (Ogawa *et al*., 2000). Ephrin A1 is also overexpressed in embryonic vasculature but is absent in mature quiescent vasculature supporting its role in neovascularization (McBride and Ruiz, 1998). *In vivo* phage display studies have revealed a variety of stage-specific molecular changes in tumor vasculature during carcinogenic progression of squamous cell carcinoma (Hoffman *et al*., 2003) and islet cell carcinoma of the pancreas (Joyce *et al*., 2003). The nature of the local regulatory signals are mostly unknown, although the specialized stroma are thought to be a major source.

Majority of the described angiogenic factors, like VEGF and angiopoietins, stimulate growth of endothelial cells in general, regardless of their tissue of origin. Endocrine-gland-derived vascular endothelial growth factor (EG-VEGF; structurally unrelated to VEGF) was identified as an endothelial cell mitogen specific for endocrine gland-derived capillary endothelial cells. It was also able to induce angiogenesis *in vivo* in endocrine tissue (LeCouter *et al*., 2001). Mouse EG-VEGF also has a role in the regulation of the phenotype and growth properties of endothelial cells in distinct capillary bed; unlike the human EG-VEGF, mouse EG-VEGF is specific for liver and kidney-derived endothelial cells (LeCouter *et al*., 2003). Two receptors for EG-VEGF have been characterized in both human and mouse, their expression patterns are commensurate to the functional effects of EG-VEGF in both human and mouse endothelial cells (Lin *et al*., 2002; LeCouter *et al*., 2003). Based on studies with CXCR4 and CXCL12 (CXCR4 ligand) deficient mice, this chemokine-chemokine receptor pair has also been suggested to act as an organ-specific angiogenesis modulator (reviewed in Romagnani *et al*., 2004).

4.3. Angiomics (functional genomics of angiogenesis)

As a result of the exploration of gene expression patterns in cancer, numerous genetic alterations associated with cancer have been found. All tumors contain genetic alterations that range from subtle changes in DNA sequence to visible cytogenetic changes like chromosome losses and rearrangements. Moreover, abnormally high genetic instability can be found in many tumors (Cahill *et al*., 1999).

Much less is known about gene expression patterns in tumor endothelia. A new area of genomics, angiomics, is now exploring this aspect of angiogenesis. By using methods like SAGE, cDNA microarrays and oligonucleotide-based microchips for analyzing differential gene expression in angiogenic vessels, angiomics will give us valuable insights into the gene expression patterns of proliferating endothelial cells in the context of the tumor microenvironment. By studying the gene expression patterns of endothelial cells derived from normal and malignant human colorectal tissues, almost 50 differentially expressed gene transcripts were found. Many of them encode ECM proteins, but most of them are still unknown. Angiomics studies have confirmed four basic hypotheses about angiogenesis. First, normal and tumor endothelia are highly related and share many endothelial cell-specific markers. Second, the endothelium derived from tumors is qualitatively different from that derived from normal tissues of the same type and is also different from primary endothelial cultures. Third, differently expressed genes are characteristically expressed in tumors from several different tissue types, demonstrating that tumor endothelium, in general, is different from the endothelium in surrounding normal tissues. Fourth, most of the genes expressed differentially in tumor endothelium are also expressed during angiogenesis of corpus luteum and wound healing. Tumors may recruit vasculature by means of the same signals elaborated during other physiological or pathological processes (St Croix *et al*., 2000).

5. Phage display technology

Phage display technology allows presentation of large peptide and protein libraries on the surface of filamentous phage permitting the selection of peptides and proteins, including antibodies, with high affinity and specificity to almost any target. This technology has had a major influence on the work and discoveries done in the fields of immunology, cell biology, drug discovery and pharmacology. The power of phage display lies in the ability to propagate selected peptide/protein ligands through multiple rounds of selection and the direct link of the phenotype of the antigen/receptor binding ligand to the genotype of the phage particle presenting the ligand.

Phage display technology was first introduced by George Smith as an expression vector, "fusion phage", capable of presenting a foreign amino acid sequence accessible to binding an antibody (Smith, 1985). Since then, large number of phage displayed peptide and protein libraries has been constructed (Bass *et al*., 1990; McCafferty *et al*., 1990; Barbas *et al*., 1991; Smith, 1991; Smith and Scott, 1993; reviewed in Hoogenboom, 2002 and Szardenings, 2003), leading to various techniques for screening such libraries. Peptide display technology has since then being applied to a wide range of protein interaction studies with purified/ recombinant proteins, cells and intact tissues *in situ* as well as *in vivo*. A vast body of work has been done using phage displayed antibody libraries for diagnostic and therapeutic applications. Since our work mainly focuses on using phage displayed peptide libraries, I will refer the reader to reviews on phage displayed antibody libraries (Rader and Barbas, 1997; Azzazy and Highsmith, 2002; Hoogenboom, 2002).

5.1. Phage display random peptide libraries

Random peptide libraries consist of large random collection of peptides, displayed as recombinant proteins on the surface of a filamentous bacteriophage. Random peptide libraries usually contain up to 10^9 individual phage clones. A comprehensive review of filamentous phage biology has been covered by Barbas *et al*. (Barbas *et al*., 2001) and will be described here only briefly.

Filamentous phage particles, known as Ff, include strains M13, f1 and ft. Fd phage particles consist of a long cylindrical protein capsid, 930 nm in length and 6.5 nm in diameter, enclosing a circular, single-stranded DNA genome of about 6400 nucleotides, consisting of 11 genes. The mass of the particle is approximately 16.3 MDa, of which 87% is contributed by protein. The length of the cylinder consists of approximately 2700 molecules of the major coat protein pVIII (encoded by the *gene VIII*) and the other end of the filamentous phage particle contains about five copies of the pIII and pVI proteins (encoded by *gene III* and *gene VI*) accounting for about 10-16 nm of the phage length and the other end contains about five copies of the pVII and pIX proteins (encoded by *gene VII* and *gene IX*). The pIII protein appears to have two functional domains: an exposed N-terminal domain that binds the F pilus, but is not required for phage particle assembly, and a C-terminal domain that is buried in the particle and is an integral part of the capsid structure. In the phage particle, the C-terminal portion of pVIII is inside the phage particle, close to the DNA, while the N-terminal part is exposed to the surroundings.

Fd particles are able to infect a variety of Gram-negative bacteria, including *E. coli,* using pili (F pilus in *E. coli*) as receptors. Fd does not produce a lytic infection in *E. coli*, instead it induces a state in which the infected bacteria produce and secrete phage particles without undergoing a lysis.

Infection is initiated by the attachment of phage pIII protein to the f pilus of *E. coli*. Only the circular single-stranded DNA enters the bacterium where it is converted by the host DNA replication machinery into the double-stranded plasmid-like replicative form. The replicative form undergoes a rolling circle replication to make single-stranded DNA and also serves as a template for the expression of pIII and pVIII proteins. Phage progeny are assembled by packaging the single-stranded DNA genome into protein coats and extruded through the bacterial membrane to the medium (Russel, 1991).

Most of the currently used phage display vectors use the N-terminus of pIII protein or pVIII protein to display the foreign peptide or protein (Smith and Scott, 1993). The pIII libraries display 3-5 copies of each individual peptide (Scott and Smith, 1990), whereas pVIII libraries can display up to 2700 copies of small (up to six amino acids) peptides (Greenwood *et al*., 1991). The pIII and pVIII proteins can display peptides of various length and cysteine residues can be introduced to the fusion peptide to create conformational constraints by the formation of "loops" between disulfide bridged cysteine residues. Furthermore, the exogenous peptides are well exposed, facilitating the insert-target interactions. Fairly large peptide inserts (up to 38 amino acids; Kay *et al*., 1993) can be introduced into the amino terminus of pIII protein without the loss of phage infectivity or particle assembly. Random peptides are encoded by synthetic oligonucleotides with each amino acid being encoded by the degenarate codon NNK (N= A, C, G or T; K= G or T). In our laboratory, we use peptide libraries where the random peptides are recombined to the amino terminus of the pIII protein using and fd-based vector, fUSE5 (Scott and Smith, 1990). These random peptide libraries are likely to yield higher affinity binding peptides, since only 3-5 copies of the fusion peptide are displayed on a given phage particle and the multivalency of the peptide is not as likely to influence the binding of the peptide to its target.

5.2. Screening phage display libraries

Phage display of random peptide libraries is a powerful method for obtaining small peptide ligands for virtually any protein of interest. A high proportion of isolated peptide ligands interact with the natural binding site of the target protein acting as antagonists or agonists of the natural protein functions (Hyde-DeRuyscher *et al*., 2000, reviewed in Kay and Hamilton, 2001). This might be because of the hydrophilic nature of the surface of the proteins, which has a role in preventing non-functional interactions with other molecules. However, the binding sites of molecules will generally allow water molecules to be displaced by the binding of specific ligands (Ringe and Mattos, 1999). Peptide ligands isolated from phage display libraries tend to react with the protein binding sites only because they are small, and will not have sufficient binding energy to displace water from the non-binding surfaces of the target protein. Besides proteins, peptides affecting biologically significant protein-DNA interactions (Cheng *et al*., 1996), peptides binding to carbohydrates (Peletskaya *et al*., 1996; Matsubara *et al*., 1999; Noda *et al*., 2001), carbon nanotubes (Wang *et al*., 2003) and small chemical compounds like taxol (Rodi *et al*., 1999) have been isolated from phage display random peptide libraries. Recently, Cardó-Vila *et al*. introduced an approach based on phage display technology to identify molecules that specifically interact with the cytoplasmic domain of the β_5 integrin and revealed a functional link between the $\alpha_v\beta_5$ integrin, annexin V, and programmed cell death (Cardo-Vila *et al*., 2003).

In general, the affinity selection of ligands from a phage display random peptide libraries involves 7 fundamental steps: i) preparation of a primary library or amplification of an existing library, ii) exposure of the phage particles to a target (immobilized protein/cell surface protein/vascular endothelium) for which specific ligands are planned to be identified, iii) removal of non-specific binders (washing/perfusion), iv) recovery of the target bound phage by elution or direct bacterial infection and amplification of the recovered phage, v) repetition of the steps i-iv, usually three to six rounds, until an enriched population of binders are obtained, and finally, vi) sequencing the peptide inserts of the enriched phage clones (Figure 4; see section 5.2.1. for a more detailed description of screening phage libraries *in vivo*). Enriched peptide inserts are then analyzed, and desired peptides can then be synthesized as recombinant or synthetic peptides for further analysis of the ligand-target interaction.

It is a common observation that the binding motif of a targeting peptide is a tripeptide motif appearing several times in different sequence contexts. Three amino acid residues seem to provide the minimal framework for structural formation and protein–protein interaction (Vendruscolo *et al*., 2001). Examples of such biochemical recognition units and binding of ligand motifs to their receptors include RGD, LDV and LLG to integrins (Koivunen *et al*., 2001; Ruoslahti, 1996), and GFE to membrane dipeptidase (Rajotte *et al*., 1998; Rajotte and Ruoslahti, 1999).

5.2.1. *In vivo* selection of phage display libraries

In vivo selection of phage display random peptide libraries allows for the selection of specific peptide ligands that bind to different vascular beds. Vascular targeting exploits molecular differences that exists in blood vessels of different organs and tissues, as well as differences between normal blood vessels and angiogenic blood vessels, for example in tumors (Pasqualini and Ruoslahti, 1996; Pasqualini *et al*., 1997; Arap *et al*., 1998; Rajotte *et al*., 1998). In the *in vivo* procedure, phage capable of homing to certain organs or tumors following an intravenous

injection are selected from the libraries; the ability of individual peptides to target a tissue can also be analyzed by this method (Pasqualini and Ruoslahti, 1996; Arap *et al*., 1998; Rajotte *et al*., 1998).

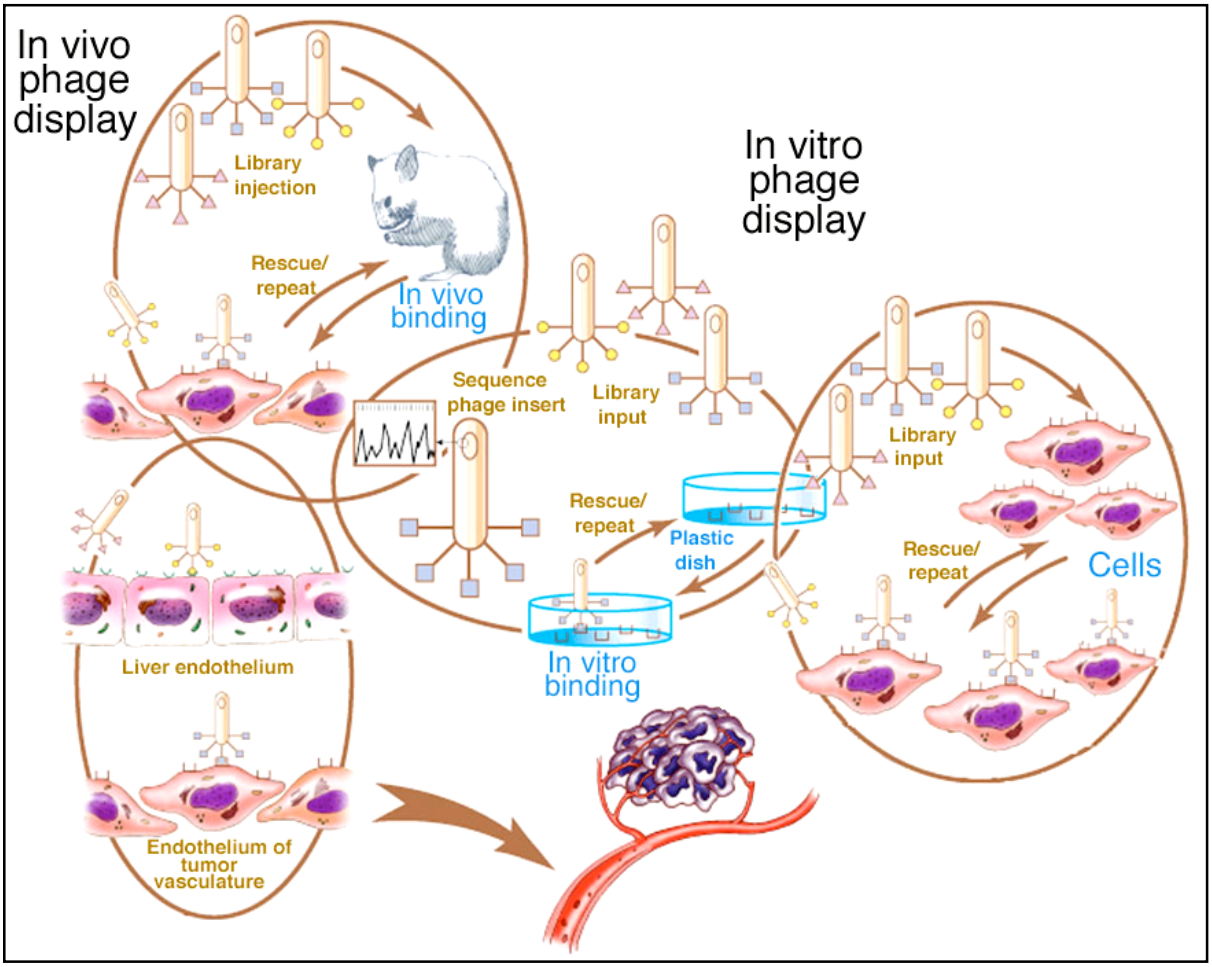

Figure 4. Schematic illustration of the selection of the phage displayed random peptide libraries for the identification of specifically binding pepdies to known and unknown targets. *In vitro* selection of peptides binding to purified receptors and cells as well as *in vivo* selection of peptides binding to specific vascular beds are illustrated.

In general, 10^9 to 10^{10} transforming units of phage displayed random peptide library are injected intravenously into a deeply anesthetized mouse via the tail vein, and phage are allowed to circulate within the mouse. Depending on the time the phage library is allowed to circulate, the selection of the peptides can be biased towards a recovery of peptides that either bind to the blood vessels (short circulation time, 5-10 minutes, before phage recovery), or peptides that will get internalized by endothelial cells of the normal blood vessels or endothelial, periendothelial or tumor cells in the tumor vasculature (circulation time 24 hours or longer). Mouse is then perfused through a catheter inserted in the left ventricle and bled through a small incision in the right atrial chamber or ascending *vena cava*. After perfusion, the desired organs are isolated, and phage are recovered from the tissue by infecting *E. coli* bacteria with the tissue homogenate, and phage are amplified. Selective binding of the phage pool is quantified by calculating the number of recovered phage titers from the organs of interest.

The amplified phage is purified and used for subsequent rounds of panning; repeated injection of phage, vascular perfusion, tissue removal and isolation and amplification of phage, until a population of phage that bind selectively to the vasculature of the target organ is obtained. Generally, three rounds of *in vivo* selection are sufficient to select for specific organ- and tumor-homing phage, but additional rounds can be done. After the desired enrichment of the phage pool to a certain organ has been obtained, 50-100 phage clones from the last two rounds of selection are sequenced. Peptide sequences are compared with each other to identify enriched peptides or peptide motifs. The selectivity of identified candidate clones is then validated by individually comparing their homing to other organs and also to the homing of an insertless phage clone (Pasqualini and Ruoslahti, 1996; Pasqualini *et al*., 2001).

Since 1996 when Pasqualini and Ruoslahti described the methodology for selecting phage capable of homing to different vascular beds by

screening of phage display random peptide libraries *in vivo* (Pasqualini and Ruoslahti, 1996), numerous murine tissue-specific endothelial cell markers have been identified this way by our laboratory and others (reviewed in Trepel *et al.*, 2002; George *et al.*, 2003). These include peptide ligands targeting brain and kidney (Pasqualini and Ruoslahti, 1996), lung, skin, pancreas, intestine, uterus, adrenal gland and retina (Rajotte *et al.*, 1998), muscle (Samoylova and Smith, 1999), prostate (Arap *et al.*, 2002), normal and malignant breast tissue (Essler and Ruoslahti, 2002), lymph nodes (Trepel *et al.*, 2001), placenta (Kolonin *et al.*, 2002) and white adipose tissue (Kolonin *et al.* 2004). *In vivo* phage display approach has also revealed a vascular address system that allows tissue-specific targeting of angiogenesis-related molecules in tumor blood vessels (Pasqualini *et al.*, 1997; Arap *et al.*, 1998; Burg *et al.*, 1999; Koivunen *et al.*, 1999; Hoffman *et al.*, 2003; Joyce *et al.*, 2003) as well as in tumor lymphatic vessels (Laakkonen *et al.*, 2002). In addition, it has been possible to identify unique molecular signatures by *in vivo* phage display that are specifically expressed at different stages of tumor progression from a highly proliferative and angiogenic dysplastic lesion to invasive phase in transgenic mouse models (Hoffman *et al.*, 2003; Joyce *et al.*, 2003). By using transgenic mouse models for multistage tumorigenesis involving the pancreatic islets of Langerhans, Joyce *et al.* identified peptides that discriminate between the vasculature of the premalignant angiogenic islets and fully developed tumors (Joyce *et al.*, 2003). Similar study has also been done with transgenic model of squamous cell carcinoma further confirming the findings of a tumor stage-specific vascular signature (Hoffman *et al.*, 2003). Peptide ligands homing to atherosclerotic regions of blood vessel walls have been identified by using *in vivo* phage display in mice deficient in low-density lipoprotein (Houston *et al.*, 2001). As a strategy to isolate peptide ligands to receptors in human synovium vasculature, Lee *et al.* have used *in vivo* phage display in conjunction with mice transplanted with human synovium tissue (Lee *et al.*, 2002). Since the blood vessels from human synovium grafts have been shown to form functional anastomosis with murine subdermal vessels and supporting the adhesion and extravasation of human leukocytes into the grafts (Wahid *et al.*, 2000), they were able to isolate peptide ligands targeting human vasculature supporting human tissue without actually using human patients (Lee *et al.*, 2002).

Integration of the isolation of ligands from the *in vivo* screenings to proteomic strategies to identify the receptors for these ligands has produced an array of tissue specific vascular receptors. Complementary approaches have been used to identify receptors for tissue homing peptides. Membrane dipeptidase on lung endothelium was identified as the receptor for GFE-peptide (Rajotte and Ruoslahti, 1999), aminopeptidase P on both normal and malignant breast tissue as the receptor for CPGPEGAGC-peptide (Essler and Ruoslahti, 2002), FcRn/β2-microglobulin as a target for the TPKTSVT-peptide in placenta (Kolonin *et al.*, 2002) and prohibitin as the receptor for CKGGRAKDC-peptide in the white adipose tissue (Kolonin *et al.*, 2004).

Besides offering a way of identifying endothelial cell surface markers accessible to the circulation and providing novel tools for selective vascular targeting, *in vivo* phage display studies further our understanding of organ and tumor endothelium specificity and define the role that endothelial cell markers play in angiogenesis. Some of these vascular markers are vascular proteases that not only serve as receptors for circulating ligands but also modulate angiogenesis (Koivunen *et al.*, 1999).

Tumor targeting peptide ligands have also been used for delivery of cytotoxic chemotherapy (Arap *et al.*, 1998), proapoptotic peptides (Arap *et al.*, 2002, Ellerby *et al.* 1999), cytokines (Curnis *et al.*, 2000) and liposomes (Pastorino *et al.*, 2003) to receptors in the angiogenic vasculature showing marked therapeutic efficacy in tumor-bearing mouse models. Tumor targeting peptide ligands can also deliver imaging agents to tumor vasculature (Hong and Clayman, 2000). There are many problems with potential therapy applications associated with the use of small peptides, including the delivery, stability and large-scale synthesis (Latham, 1999); however, they can be an excellent starting point for rational design of drug candidates. Peptidomimetics can be designed maintaining the overall structure and biological properties of the peptide, while increasing the stability of the peptidomimetic compounds (Kieber-Emmons *et al.*, 1997; Ripka and Rich, 1998).

Aims of the study

The specific aims of this study were

1) To identify the receptor for the tumor homing NGR-peptide (Arap *et al.*, 1998) and to study the functional role of this receptor (CD13/ aminopeptidase N) in angiogenesis.
2) To study the role of aminopeptidase A (APA) in angiogenesis and identify APA ligands that can be used for targeted therapy.
3) To examine whether reducing the metabolic rate of the retina by abrogating photoreceptor cells will functionally affect angiogenesis in the retina.
4) To develop new methodology for the identification of functional molecular targets on blood vessels.

Materials and methods

Detailed descriptions of the materials and methods are found in the original publications.

Materials	**Original publication**
Animals	I, II, III, IV
Antibodies	I, II, III, V, VI
Cell lines	I, II, III, V
cDNA probes	III, IV
Chemical enzyme inhibitors	II, III
Patients	IV, VI
Phage libraries	I, V, VI
Plasmids	I, II, III
Purified proteins, growth factors and receptors	I, II, III, V, VI
Synthetic peptides	I, II, V, VI
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Results

1. CD13/APN and APA as functional targets in angiogenic blood vessels (I-III)

1.1. CD13/APN and APA expression in angiogenic vasculature (I, II)

Pasqualini *et al*. previously identified the RGD-binding peptide motif, WN/DDGWL (Pasqualini *et al*., 1995). This motif resembles a site in certain integrin ß subunits that participates in binding RGD-containing integrin ligands. This motif is also present in the extracellular domain of CD13/APN and some other aminopeptidases. Given the similarity of the RGD and NGR motifs, we hypothesized that the receptor for the NGR phage in tumor vasculature (Arap *et al*., 1998) might be an aminopeptidase.

We studied the expression of CD13/APN in endothelial cells to determine whether its expression is consistent with its role as the homing receptor in tumors for the NGR phage. Immunohistochemical staining showed strong mouse CD13/APN expression in the vasculature of tumors formed by the MDA-MB-435 human breast carcinoma cells in nude mice (II, Fig. 3A). An antibody specific for human CD13/APN did not stain these tumors (II, Fig. 3B), which agrees with the lack of CD13/APN expression by these cells *in vitro*. The blood vessels in all normal mouse tissues examined were negative for CD13/APN (II, Fig. 3C and 3D show liver and spleen, respectively).

APN immunostaining was also found in blood vessels, including capillaries, of various tumors from patients. Fig. 4A (II) shows vascular CD13/APN staining in a human breast carcinoma. The vasculature in human malignant gliomas and lymph node metastases from multiple tumor types was also positive for CD13/APN. In contrast, the blood vessels in various normal human tissues were essentially negative for CD13/APN. Faint staining was sometimes seen in endothelial cells of arteries but not in capillaries; Fig. 4B (II) shows such staining for normal breast tissue. Blood vessels in corpus luteum expressed CD13/APN (II, Fig. 4C). Consistent with the fact that blood vessels in this tissue undergo angiogenesis (Klauber *et al*., 1997), this finding suggests that the expression of CD13/APN in blood vessels is related to angiogenesis.

Confocal immunofluorescence microscopy showed that endothelial cells and periendothelial layers of the vessels (presumably pericytes) expressed CD13/APN in tumors (II, Fig. 4D). The periendothelial CD13/APN staining co-localized with staining for APA, a marker of pericytes and smooth muscle cells (Schlingemann *et al*., 1996) in blood vessels. In the described tissue localization studies, two different antibodies, one against human CD13/APN (WM15) and the other against mouse CD13/APN (R3-63), were used. Both showed similar localization of CD13/APN in human and mouse tissues.

The extensive proliferation of blood vessels in malignant gliomas suggests an early role of angiogenesis in brain tumors (Wesseling *et al*., 1995). To investigate the expression of APA in brain tumor vasculature, we compared the localization of APA expression by immunostaining to the localization of APA activity by enzyme histochemistry in a panel of primary and metastatic human brain tumors and in the incidental normal tissue adjacent to the tumors. APA enzyme activity in tissue sections co-localized with APA expression as detected by an RC38 antibody. The staining pattern was consistent with APA localization to perivascular cells in the tumor microvasculature (I, Fig. 5A and 5B, glioblastoma multiforme; 5C and 5D, squamous cell carcinoma of the lung metastatic to the brain). Staining and enzymatic activity were apparent in sites of glomerular vascular proliferation in malignant gliomas and metastatic carcinomas. In contrast, APA expression and activity were barely detectable in capillaries in normal brain parenchyma. By double-staining with RC38 and with a PAL-E antibody, a general endothelial marker (Schlingemann *et al*., 1985), APA expression in colon adenocarcinoma metastatic to the brain appears to be restricted to perivascular cells (I, Fig. 5E). We also showed by immunostaining that blood vessels in human non-malignant granulation tissue express APA (I, Fig. 5F). Finally, we localized APA staining to perivascular cells by immuno-electron microscopy (I, Fig. 5G).

These results show that CD13/APN and APA are present in tumor blood vessels of various tumors from patients. Furthermore, APA present in the blood vessels during the progression of malignant tumors is enzymatically active and therefore may contribute to the angiogenic process associated with human brain tumors.

1.2. CD13/APN and APA binding peptides derived from phage display random peptide libraries (I, II)

We tested the binding of NGR phage to immunocaptured CD13/APN. Two different NGR phage bound specifically to immunocaptured CD13/APN, whereas the tumor-homing RGD-4C phage and another RGD phage showed no binding (II, Fig. 1A). The specificity of the binding was further examined by inhibiting the CD13/APN binding of one of the NGR phage with soluble peptides. Addition of the soluble, cyclic CNGRC peptide blocked the binding of NGR phage to CD13/APN while two other cyclic control peptides had no effect (II, Fig. 1B).

NGR phage also bound to Molt-4 T-cell leukemia and MDA-MB-435 breast carcinoma cells transfected with CD13/APN cDNA (Ashmun and Look, 1990), but not cells transfected with empty vector or with CD20 cDNA (II, Fig. 1C). The binding of NGR phage to the cells was blocked by the CNGRC peptide in a dose-dependent manner, but not by a control peptide of a similar general

structure (CARAC). Furthermore, a conjugate of the CNGRC peptide and the cytotoxic drug doxorubicin was selectively toxic to CD13/APN-expressing cells. When CD13/APN-transfected and control MDA-MB-435 cells were exposed to the conjugate for only 20 minutes, a significant fraction of the CD13/APN-expressing cells were killed, while CD13/APN-negative cells were essentially unaffected (II, Fig. 2). Free doxorubicin and doxorubicin coupled to the CARAC control peptide showed no significant toxicity upon incubation of these cells for the same period of time. The doxorubicin-RGD-4C conjugate was toxic to cells, regardless of their CD13/APN expression. This outcome is in agreement with the expression of the $\alpha_v ß_3$ integrin by the MDA-MB-435 cells (Arap *et al.*, 1998). The selective toxicity of the CNGRC conjugate was lost when the incubation with the drug was prolonged; upon prolonged exposure, all doxorubicin compounds were found to be highly toxic to both types of cells. These results provide further evidence for the binding of NGR to CD13/APN at cell surfaces.

To identify peptides capable of binding to APA, we screened APA-transfected cells with a phage display library (Smith and Scott, 1993). We stably transfected SK-RC-49 renal carcinoma cells with a vector expressing full-length APA cDNA. We verified that APA was functional in transfected cells by an enzyme activity assay specific for APA. An increase in phage binding to SK-RC-49/APA cells relative to SK-RC-49 cells was observed in the third round of selection (I, Fig. 2A). DNA sequencing revealed an enrichment of the sequence CYNLCIRECESIC-GADGA-CWTWCADGCSRSC containing tandem repeats of the general library sequence $CX_3CX_3CX_3C$ on each side of the pIII peptide linker GADGA sequence. 50% of randomly selected phage clones displayed such tandem repeat after the second round and 100% displayed it after the third round (I, Table 1).

Phage displaying enriched peptide motifs were tested individually for APA binding. Phage displaying the inserts CPKVCPRECESNC, CYNLCIRECESICGADGACWTWCADGCSRS C or CLGQCASICVNDC preferentially bound to SK-RC-49/APA cells relative to SK-RC-49 cells; insertless control phage (fd-tet) showed no binding preference (I, Fig. 2B). We synthesized peptides containing APA-binding motifs (consensus sequence CPRECESIC; I, Table 1). Specificity of the binding of phage displaying C P K V C P R E C E S N C or CYNLCIRECESICGADGACWTWCADGCSRS C peptides to SK-RC-49/APA cells was demonstrated by inhibition of phage binding by the synthetic peptide; several negative control peptides had no inhibitory effect. Binding of CLGQCASICVNDC phage to SK-RC-49/APA cells was not affected by the synthetic peptide suggesting that the SIC sequence in the consensus motif is not critical for binding. We also evaluated whether the selected peptides would bind to the isolated APA protein. We immunocaptured APA from SK-RC-49/APA lysate with the RC38 antibody and showed, that it remained enzymatically active (I, Fig. 2C). We used phage displaying CPKVCPRECESNC, C L G Q C A S I C V N D C, or CYNLCIRECESICGADGACWTWCADGCSRS C in binding assays to the immunocaptured APA. We found up to 12-fold enrichment of phage binding to APA compared to immunocaptured cell lysates from the SK-RC-49 cells; control insertless phage (fd-tet) showed no binding preference (I, Fig. 2D).

We also wanted to evaluate whether the synthetic CPRECESIC peptide had any effect on APA enzyme activity. We incubated APA-transfected cells with the APA-specific substrate α-glutamyl-p-nitroanilide with increasing concentrations of APA-binding or control peptides. The APA-binding peptide inhibited APA enzyme activity while the control peptide had no effect (I, Fig. 3A). We calculated the IC_{50} of the peptide for APA enzyme inhibition to be ~800 μM. The inhibition was specific to APA since the peptide did not affect the enzymatic activity of the closely related protein CD13/APN.

These data support the specific binding of the CNGRC peptide to CD13/APN and peptides with the consensus motif CPRECESIC to APA.

1.3 CD13/APN and APA as targets for systemic delivery of therapeutics to angiogenic vasculature (I, II)

To assess the possibility of using CD13/APN and APA as vascular targets for systemic delivery of therapeutics to tumors, we evaluated the ability of APA and CD13/APN binding phage to home to tumor blood vessels *in vivo*.

10^9 TU/mouse of CD13/APN binding CNGRC -phage were administered intravenously to nude mice bearing human breast carcinoma MDA-MB-435-derived xenografts. Phage homing was quantified by recovery from tissue homogenates by bacterial infection. CNGRC phage was enriched in tumors compared to a control insertless phage (fd-tet) In addition, the *in vivo* homing of the CNGRC phage to tumors was blocked by co-injection of a rat anti-mouse CD13/APN antibody R3-63 (II, Fig. 1D) capable of inhibiting the enzymatic activity of CD13/APN (Hansen *et al.*, 1993). In contrast, tumor homing of the RGD-4C phage was not affected by R3-63, and normal rat IgG had no effect on the homing of either phage. (II, Fig. 1D).

Similarly, 10^9 TU/mouse of the APA binding CPKVCPRECESNC phage were administered intravenously to nude mice bearing human breast carcinoma MDA-MB-435-derived xenografts. Phage homing was quantified by recovery from tissue homogenates by bacterial infection. CPKVCPRECESNC phage was enriched in tumors compared to control organs; in contrast, control insertless phage (fd-tet) showed no enrichment in tumors (I, Fig. 6A). Homing of

CPKVCPRECESNC phage to tumor vasculature was subsequently confirmed by immunohistochemical staining of phage on tissue sections. Strong phage staining was observed in tumor blood vessels, but not in normal brain blood vessels, of mice that received CPKVCPRECESNC phage; in contrast, control phage without insert did not home to tumor blood vessels (I, Fig. 6B).

These results show that CD13/APN and APA-binding phage target tumor blood vessels *in vivo*. Thus both CD13/APN and APA expressed in the angiogenic vasculature can serve as targets for systemic delivery of therapeutics.

1.4. Inhibition of angiogenesis and tumor growth by genetic elimination or biochemical inhibition of CD13/APN and APA (I, II)

To test the functional role of the CD13/APN and APA enzymatic activities in angiogenesis, we studied the effects of CD13/APN and APA inhibitors in the chick embryo chorioallantoic membrane (CAM) assay. In this simplified *in vivo* model of angiogenesis, neovascularization is stimulated with VEGF of bFGF adsorbed on to a gelatin sponge placed on the CAM (Ribatti *et al.*, 1997).

Immunostaining of the CAM showed that the anti-mouse CD13/APN antibody R3-63 recognizes CAM (chicken) vasculature, making it possible to test its effect on bFGF-induced angiogenesis in the CAM. CAMs were stimulated on embryonic day (E) 8 with sponges containing bFGF only, bFGF plus anti-APN R3-63, bFGF plus normal rat IgG, bFGF plus bestatin, bFGF plus actinonin, or bFGF plus leupeptin. CAMs were examined on E12 and neovascularization was quantified by counting the number of emerging capillaries. R3-63 significantly suppressed vessel growth in the CAM assay, as did CD13/APN inhibitor bestatin and actinonin, another chemical CD13/APN inhibitor (II, Fig. 5B). Normal rat IgG or leupeptin, which does not inhibit CD13/APN activity, had no effect (II, Fig. 5B). Similarly, CAMs were stimulated with sponges containing VEGF only, VEGF plus CPRECESIC, or VEGF plus control peptides. CAMs were examined and neovascularization was quantified (I, Fig. 4B). Significant induction of neovascularization was found when a sponge with VEGF was placed on the CAM compared to a sponge with no growth factors. CPRECESIC peptide (1 mM) inhibited VEGF-induced CAM neovascularization by 40%. Equimolar concentrations of a negative control peptide or lower CPRECESIC peptide concentrations (0.1 mM and 0.3 mM) did not affect the number or branching of the growing blood vessels.

These data show that inhibiting the enzymatic activity of either CD13/APN or APA inhibits blood vessel formation in a standard model of angiogenesis.

We used the mouse model of O_2-induced retinopathy (Smith *et al.*, 1994) to test the effect of biochemical CD13/APN inhibition on angiogenesis. Mice were exposed to 75% O_2 from P7 to P12, and then returned to room air and treated with intravenous injection of vehicle alone, anti-mouse CD13/APN R3-63 and 2M-7 antibodies or normal rat IgG (250 μg/mouse), or bestatin, (200 μg/mouse). Angiogenesis was quantified by counting endothelial cell nuclei protruding into the vitreous space on P17. The number of retinal neovessels in mice treated with vehicle alone was set at 100%. Both anti-mouse CD13/APN antibodies and bestatin reduced the neovascularization significantly, whereas rat IgG had no effect (II, Fig. 5A).

To evaluate whether CD13/APN inhibitors (chemical CD13/APN inhibitor bestatin or anti-APN blocking antibodies) suppress tumor growth *in vivo*, we used MDA-MB-435 derived breast carcinoma xenografts. We chose this model because CD13/APN expression is undetectable in MDA-MB-435 cells, but strongly induced in the tumor blood vessels associated with MDA-MB-435 -derived tumors (II, Fig. 3A) and accessible upon systemic delivery as shown by the CNGRC-phage homing to the tumor vasculature (Arap *et al.*, 1998; II, Fig. 1D). Tumor-bearing mice received vehicle, bestatin (250 μg/mouse), a mixture of R3-63 and 2M-7 antibodies, or normal rat IgG (125 μg/mouse) given intraperitoneally once a week for 3 weeks and tumors were monitored for three weeks. Anti-mouse CD13/APN antibodies and bestatin inhibited significantly the growth of tumors, whereas normal rat IgG had no effect (II, Fig. 5C).

To evaluate whether APA inhibitory ligands (APA-binding peptides or anti-APA blocking antibodies) suppress tumor growth *in vivo*, we used EF43-*fgf4*-derived tumors, an established mouse mammary carcinoma model (Deroanne *et al.*, 1997; Hajitou *et al.*, 2001). We chose this model because APA expression is undetectable in EF43-*fgf4* cells but strongly induced in the tumor blood vessels associated with EF43-*fgf4*-derived tumors (I, Fig. 7 A) and homing of APA-binding phage to tumor vasculature was experimentally validated. Tumor-bearing mice received vehicle, CPRECESIC or control peptides (250 μg/mouse) and tumors were monitored (I, Fig. 7B). We observed differences in tumor growth as early as 5 days after treatment initiation and when the experiment was terminated at the end of two weeks, CPRECESIC-treated mice had significantly smaller tumors relative to tumor-bearing control mice that received control peptide; tumors in mice treated by control peptide behaved similarly to those tumors in mice receiving vehicle alone indicating that the control peptide had no measurable effect. However, because the APA-inhibitory peptides may require a relatively high molar range (~250 nanomoles/mouse/dose) to be effective, we also evaluated the effects of anti-APA antibodies on tumor growth. We used an established regimen of two anti-APA monoclonal antibodies (ASD-37/41) with

synergistic APA inhibitory properties (Assmann *et al*., 1992; Mentzel *et al*., 1999) on the same animal model. Tumor-bearing mice received vehicle, ASD-37/41, or isotype control IgG (200 µg/mouse) and tumors were monitored (I, Fig. 7C). Again, we noticed clear differences in tumor growth. By the end of two weeks when the experiment was terminated, ASD-37/41-treated mice had significantly smaller tumor volumes relative to tumor-bearing control mice that received isotype negative control IgG; tumors in mice treated with control IgG behaved similarly to those tumors in mice receiving vehicle only indicating that control IgG had no detectable effect on tumor growth. Finally, to analyze post-treatment effects of APA-inhibitory antibodies in tumors and their angiogenic blood vessels, we examined tissue sections of all groups by CD31-immunostaining (I, Fig. 7D). In contrast to the recognized (Deroanne *et al*., 1997; Hajitou *et al*., 2001) extensive blood vessel network typically observed in EF43-*fgf4*-derived tumors, ASD-37/41-treated tumors exhibited a mixture of viable tissue (towards the outer rim of the tumors) and neighbouring regions of disrupted vascular structure along with large areas of widespread cell death and destruction of the tumor architecture; inhibitory peptides had similar but less pronounced effect on treated tumors (I, Fig. 7D).

Taken together, these results indicate that CD13/APN and APA-inhibitory ligands and antibodies can specifically target angiogenic vasculature and suppress tumor growth *in vivo.*

We have also evaluated the angiogenic response of APA -/- mice in two *in vivo* models of angiogenesis. First, we used a mouse model of O_2-induced retinopathy (Smith *et al*., 1994). APA wild type (APA +/+), APA heterozygotes (APA+/-) and APA-null (APA -/-) mice were exposed to 75% O_2 from P7 to P12, and then returned to room air. Retinas were analyzed at P17, when neovascularization had supervened on the vitreal surface. Angiogenesis was quantified by counting endothelial cell nuclei protruding into the vitreous space (Smith *et al*., 1994). Induction of retinal angiogenesis was seen in wild type mice on P17 (I, Fig. 1A), but significantly fewer endothelial cell nuclei were found in retinas from APA -/- mice (I, Fig. 1B). An intermediate decrease in retinal angiogenesis was observed in the APA +/- mice suggesting dose-dependence (I, Fig. 1C). No endothelial cell nuclei protruding into the vitreous space were found in mice exposed only to room air regardless of their genotype or time of analysis.

As a second model, we adopted a quantitative *in vivo* angiogenesis assay utilizing implanted gelfoam sponges saturated with angiogenic growth factors VEGF, bFGF and TGF-α (McCarty *et al*., 2002). To compare angiogenesis induction from subcutaneous tissue, sponges were implanted into the flanks of wild type (APA +/+) or APA-null mice (APA -/-). After two weeks, the sponges were harvested and stained with anti-CD31 antibodies to detect the endothelial cells of newly formed blood vessels permeating the sponge. Strong induction of CD31-positive capillary structure formation was observed in the growth factor-absorbed sponges implanted into wild type mice (I, Fig. 1D) whereas the number of CD31-positive capillary structures was much smaller in APA -/- mice and the only limited capillary formation observed was observed at the outer surface of the sponge (I, Fig. 1E).

These results suggest a deficient angiogenic response in APA -/- mice to relative hypoxia or to angiogenic growth factors as compared with their otherwise isogenic APA +/+ counterparts.

1.5. CD13/APN activation by angiogenic signals (III)

To investigate the mechanisms of CD13/APN induction in tumor vasculature, the regulation of CD13/APN by factors contributing to angiogenic progression were studied.

First, we characterized *CD13/APN* endothelial cell transcripts by Northern blot analysis. *CD13/APN* cell-surface expression is regulated by two independent, mutually exclusive, promoters separated by an 8-kb intron (Shapiro *et al*., 1991; III, Fig. 1A). Analysis of *CD13/APN* transcripts in primary HUVECs or the Kaposi sarcoma-derived KS1767 cell line by Northern blot demonstrated that the endothelial cells express the shorter 3.4-kb transcript (III, Fig. 1B). These data suggest that *CD13/APN* transcription in endothelial cells initiates from the proximal liver/intestinal epithelial promoter and that the KS1767 cell line faithfully recapitulates *CD13/APN* transcription initiation in primary cells. Further analysis by S1 nuclease, RT-PCR, and immunoprecipitation confirmed that the *CD13/APN* transcript in KS1767 cells initiates from the proximal start site, is identical in sequence to that expressed in hepatic epithelial cells, and encodes a protein that is indistinguishable in size and abundance from that expressed in hepatocytes and myeloid cells.

In order to delineate the promoter region regulating endothelial cell expression, we fused approximately 1-kb of upstream sequences flanking the transcriptional start site of either the distal (myeloid) or proximal (epithelial) promoter (III, Fig. 1A) upstream of the CAT or luciferase reporter genes and transiently transfected these constructs into HUVECs or KS1767 cells (III, Fig. 2A and B, respectively). Significant reporter gene activity was observed in those cells containing sequences from the proximal, but not the distal, promoter, indicating that the information required for *CD13/APN* expression in both primary endothelial cells and cell lines is contained within this 1-kb fragment.

To investigate whether hypoxia induces *CD13/APN* expression, we evaluated the response of endogenous *CD13/APN* mRNA and the proximal promoter constructs to either hypoxic culture conditions or cobalt chloride treatment. RT-PCR analysis indicated that the expression of

CD13/APN increases *in vivo* in mouse retinas undergoing hypoxia-induced neovascularization when compared with untreated controls (III, Fig. 3A). In addition, treatment of KS1767 cells with cobalt chloride or in environmental hypoxia induced a time-dependent expression of endogenous *CD13/APN* mRNA and protein (III, Fig. 3B and C) and transfected *CD13/APN* reporter gene levels (III, Fig. 3D). Therefore, hypoxia increases *CD13/APN* expression in a manner analogous to the hypoxic transcriptional up-regulation observed with other angiogenic regulators (Folkman and D'Amore, 1996; Hanahan and Folkman, 1996; Hanahan, 1997).

To address the possibility that the hypoxic induction of *CD13/APN* is indirectly mediated by hypoxia-induced growth factor up-regulation in vascular endothelium, we cultured primary HUVECs in low or high concentrations of serum (which contains functional concentrations of many angiogenic factors) or with the individual angiogenic growth factors, bFGF, VEGF, TNF, or IGF-1 (III, Fig. 4A). *CD13/APN* expression was significantly induced upon serum stimulation (4-fold) and increased between 1.5- to 2.5-fold on culture with each of the individual angiogenic factors (bFGF > VEGF = TNF > IGF-1). Similarly, treatment of HUVECs with these same angiogenic factors up-regulated levels of the 3.4-kb CD13/APN transcript to varying degrees (III, Fig. 4B), implying that transcription from the proximal promoter is induced by certain angiogenic growth factors. Therefore, the expression of *CD13/APN* is activated in primary endothelial cells by growth factors that are produced in the tumor microenvironment.

To confirm that our *in vitro* model faithfully reflects the induction of CD13/APN in primary vascular endothelium, we assessed whether *CD13/APN* expression in the KS1767 cell line was also serum regulated. Flow cytometric analysis of KS1767 cells cultured in the presence of 10% serum for 24 hours demonstrated a 10-fold increase in CD13/APN cell surface expression when compared with cells exposed to low-serum concentrations (III, Fig. 5A). Similarly, Northern analysis of KS1767 cells showed a dose-dependent 3- to 4-fold increase in *CD13/APN* message levels in response to increasing levels of serum (and consequently, angiogenic factors, III, Fig. 5B), as well as a time-dependent response to the individual factors bFGF and VEGF (Fig. 5C), comparable with that of primary vascular endothelium (III, Fig. 4B). Therefore, these data confirmed the validity of the KS1767 cell line as an *in vitro* model for the study of *CD13/APN* regulation in endothelial cells.

To confirm, that the proximal promoter controls expression of *CD13/APN* during angiogenesis, we examined whether a reporter plasmids containing this promoter is also be upregulated in response to serum and growth factor stimulation. KS1767 cells were transiently transfected with a proximal promoter-driven luciferase reporter plasmid and cultured in medium containing 1% serum and either bFGF, VEGF, or IGF-1 (III, Fig. 6A). The bFGF-treated cells consistently showed a 2.5-fold increase in promoter activity, whereas the addition of VEGF or TNF resulted in a more modest, 1.5-fold increases over controls. Simultaneous addition of bFGF and VEGF produced a less than additive effect, perhaps suggesting a functional overlap between the stimulatory effects of the individual factors. Importantly, minimal induction was observed with those conditions containing IGF-1, demonstrating that the response is specific for certain angiogenic growth factors. To confirm these observations, neutralizing antibodies for human bFGF, human VEGF, or both factors were added to cultures of KS1767 cells transiently transfected with the proximal promoter constructs in 10% serum; this resulted in a progressive inhibition of *CD13* promoter activity (III, Fig. 6B). Together, these results indicate that the proximal promoter regulates *CD13/APN* transcription in response to angiogenic growth factors, and is therefore likely to control *CD13/APN* expression during angiogenesis.

1.6. CD13/APN and APA as regulators of endothelial cell function and morphogenesis (I, III)

We studied the regulation of CD13/APN expression on endothelial cells differentiating into capillary-like structures characteristic of angiogenesis on Matrigel to determine whether our promoter data obtained in KS1767 cells accurately reflect transcriptional regulation during angiogenesis. To ensure that the Matrigel-model recapitulated the pattern of selective expression of *CD13/APN* in tumor endothelium (II, Fig. 4A and D), we analyzed the RNA from matrix-stimulated or matrix-unstimulated EOMA cells (Obeso *et al.*, 1990). EOMA cells express very low levels of CD13/APN (III, Fig. 1) and, in this respect, correspond to resting endothelial cells. RT-PCR analysis indicated that the low endogenous *CD13/APN* mRNA levels in EOMA cells are increased 3-to 4-fold on angiogenic stimulation, similar to the levels of the angiogenic regulator β_3 integrin, whereas control β-actin levels remain unchanged (III, Fig. 7A).

To confirm that the proximal *CD13/APN* promoter controls its expression during angiogenesis, we stably transfected reporter plasmids containing this promoter upstream of the GFP reporter gene into EOMA cells. Stably transfected cell pools in cultured under unstimulated conditions formed a cobblestone monolayer characteristic of endothelial cells and were negative for CD13-driven GFP expression throughout the culture period, arguing against inhibition of expression via cell-cell contact (III, Fig. 7B). In contrast, upon angiogenic stimulation on the Matrigel, the CD13-GFP-containing transfectants formed extensive capillary networks that were highly fluorescent, confirming that the factors regulating this promoter are responsible for the induction of *CD13/APN* gene expression during tube formation.

We injected the GFP-containing EOMA cell lines subcutaneously into immuno-compromised mice to form tumor xenografts in order to establish that the proximal promoter responds to angiogenic signals during tumor progression *in vivo*. Tumors were harvested, mechanically disrupted to form single-cell suspensions, and assayed for GFP expression by flow cytometric analysis. Tumors derived from the CMV-GFP- and CD13-GFP-containing EOMA lines contained 23% to 30% GFP-positive cells compared with background levels found in the null-GFP-derived tumors (III, Fig. 7C). The relatively low percentage of GFP-positive cells in the xenografts reflects a prominent host-derived stromal component of these tumors. Therefore, transcription from the *CD13/APN* proximal promoter plasmid faithfully reflects the up-regulation of the endogenous *CD13/APN* gene that occurs during endothelial cell capillary tube formation *in vitro* and tumor growth *in vivo.*

Because *CD13/APN* transcription is induced in endothelial cells undergoing capillary tube formation, it is possible that its functional role is at the level of control of endothelial morphogenesis. To address this possibility, we cultured primary HUVECs on Matrigel in the presence of the CD13/APN inhibitors, bestatin or amastatin, or with anti-APN blocking antibody (MY7). Each of the inhibitors was extremely effective at abrogating the ability of the cells to organize a capillary network; under control conditions with vehicle alone, a nonspecific protease inhibitor (trypsin inhibitor) or isotype-matched control antibodies (UPC10) formed characteristic, organized networks (III, Fig. 8A). In contrast, treatment of HUVECs with MY7 or bestatin had no effect on proliferation rates as measured by metabolic activity (III, Figure 8B), implying that in contrast to APA, CD13/APN does not play a role in endothelial cell proliferation during angiogenesis.

Because of the enzymatic inhibitory activity of APA-binding peptides and the reduced tumor growth in mice treated with APA inhibitors, we reasoned that the APA inhibitory peptide might affect endothelial cell function. We correlated the effect of APA inhibition by the CPRECESIC peptide on the migration and proliferation of endothelial cells. For this study, we used human dermal microvascular endothelial cells (HMEC) in which the APA expression was verified by immunostaining with RC38 and the APA activity by its specific enzyme assay. CPRECESIC peptide suppressed the migration of HMECs in the Boyden chamber assay (I, Fig. 3B). Suppression of cell migration was dose-dependent and commensurate with inhibition of APA enzymatic activity. Up to 60% suppression in cell migration was observed at 1 mM of peptide but significant inhibition was detected at concentrations as low as 100 μM (I, Fig. 3B). Similarly, proliferation of HMECs was suppressed by CPRECESIC (~40% at 1 mM) in a dose-dependent manner (I, Fig. 3C). Inhibition of cell proliferation was also observed at 0.3 mM but lower peptide concentrations had no significant effect. A negative control peptide affected neither cell migration nor proliferation.

To determine whether inhibition of APA enzyme activity with CPRECESIC peptide would affect endothelial cell morphogenesis and capillary tube formation, we tested the ability of HMECs to differentiate into the capillary-like structures characteristic of angiogenesis in the presence of CPRECESIC on a gel of reconstituted basement membrane matrix (Matrigel). The formation of cord/tube-like structures was progressively impaired by increasing concentrations of CPRECESIC peptide, relative to the network formation in controls without peptide (I, Fig. 4A, upper left panel) or with an unrelated control peptide (Fig. 4A, upper right panel). The number and length of capillary-like branching structures was reduced at 0.3 mM CPRECESIC peptide (I, Fig. 4A, lower left panel), whereas cell interconnections and the capillary network organization were severely lost at a peptide concentration of 1mM (I, Fig. 4A, lower right panel).

2. An anti-angiogenic state in the retinas of mice and humans with photoreceptor cell degeneration (IV)

2.1. Absence of angiogenesis and the failure of up-regulation of VEGF in mice with inherited retinal degeneration (IV)

To test our hypothesis whether O_2 consumption by rod cells is a major driving force in ischemic retinal neovascularization, we designed experiments to test the angiogenic response of the *Pdeb*^{rd1} mutant retinas in response to ischemia by using the mouse models of O_2-induced retinopathy (Smith *et al.*, 1994) and retinal degeneration (Sidman and Green, 1965) simultaneously. *Pdeb*^{rd1} mice have a classic autosomal recessive inherited degenerative disease of photoreceptor cells known as retinal degeneration. This disease is caused by a nonsense mutation in the β subunit of the rod photoreceptor cell-specific phosphodiesterase (Sidman and Greene, 1965; Bowes et al., 1990; Pittler and Baehr, 1991; Lem et al., 1992). Light absorption by rhodopsin activates transducin, a G-protein, which in turn promotes cGMP hydrolysis by the specific phosphodiesterase, leading to hyperpolarization of rod photoreceptor cells (Roof and Makino, 2000). The retinal development in *Pdeb*^{rd1} / *Pdeb*^{rd1} mice proceeds normally until P11. At that time development of photoreceptor cell outer segments arrests and the rod cell nuclei, inner segments and outer segments begin to degenerate. Photoreceptor cell degeneration then proceeds rapidly, and exceeds 80% by P15, and 90% by P21 (Farber, 1995). By P25-30 only one sparsely populated row of photoreceptor cell nuclei remains and the outer segments have disappeared. By the beginning of the fourth postnatal week, most surviving photoreceptor cells are cone cells (Carter-Dawson et al., 1978). Apoptosis of the photoreceptor cell is the final

pathogenic event common to all animal models of retinal degeneration (Chang et al., 1993; Portera-Cailliau et al., 1994). Combination of the O_2-induced retinopathy and retinal degeneration produced the surprising finding that the reactive retinal neovascularization characteristic of normal young mice exposed to high O_2 levels, and observed in wild-type (wt) and heterozygous animals, failed to occur in *Pdeb*^{rd1} homozygotes. Neovascularization was quantified by counting vascular endothelial cell nuclei protruding into the vitreous space from at least six sections of 8-36 eyes in five independent experiments (IV, Table 1). Extensive induction of retinal neovascularization was seen in C57BL/6 +/+ wt mice on P17 after 75% oxygen treatment from P7 to P12 (IV, Fig.1B) and in heterozygous +/ *Pdeb*^{rd1} mice. Virtually no endothelial cell nuclei were seen in the *Pdeb*^{rd1}/*Pdeb*^{rd1} retinas on P17 after exposure to 75% oxygen from P7 to P12 (IV, Fig. 1D). At this time only a few layers of nuclei remained in the photoreceptor cell layer. Also, no endothelial cell nuclei were seen on or after P21, ruling out the possibility of delayed retinal neovascularization. No endothelial cell nuclei were seen on P17 in either wt or *Pdeb*^{rd1}/*Pdeb*^{rd1} mice exposed only to room air (IV, Figs. 1A and C). Staining for von Willebrandt Factor confirmed that the cells protruding into the vitreous of wt mice treated with 75% oxygen were indeed endothelial cells (IV, Fig. 1E and G) and that such cells were almost completely confined to the neural retina in *Pdeb*^{rd1}/*Pdeb*^{rd1} homozygotes (IV, Fig. 1G and H).

VEGF has been suggested to be one of the key angiogenic factors in oxygen-induced retinal neovascularization (Alon *et al*., 1995; Pierce *et al*., 1995; Stone *et al*., 1995; Okamoto *et al*., 1997; Duh and Aiello, 1999). We hypothesized that differences in VEGF expression could play a role in the lack of neovascularization in the retinas of *Pdeb*^{rd1}/ *Pdeb*^{rd1} mice and therefore examined VEGF-expression in retinal tissue by Northern blot analysis (IV, Fig. 2). Total RNAs from wt and *Pdeb*^{rd1}/ *Pdeb*^{rd1} mouse retinas were analyzed on P12 after exposing mice for 5 days to either 75% O_2 or to room air. A decline in VEGF expression was seen during exposure to hyperoxia. This decrease was followed by a 150% increase in the VEGF expression in wt mouse retinas observed 12 hours after the return to room air after 75% O_2 exposure, compared to that seen following exposure to room air only. In *Pdeb*^{rd1}/ *Pdeb*^{rd1} mice retinal VEGF expression remained low and unchanged even after exposure to 75% O_2 for 5 days, comparable to retinas of similar (otherwise isogenic) mice exposed only to room air. To determine whether the inhibition of neovascularization was a consequence of an altered spatial expression pattern of VEGF rather than an overall alteration in VEGF expression levels in the *Pdeb*^{rd1}/ *Pdeb*^{rd1} mouse retina, we analyzed VEGF-expression in the retina by in situ hybridization. Tissue sections from wt and *Pdeb*^{rd1}/ *Pdeb*^{rd1} mouse eyes were evaluatedonP12, 12 hours after of exposure to either 75% O_2 or room air for 5 days. Slightly higher VEGF mRNA levels were seen in the inner nuclear layer and in the inner plexiform layer of wt mouse retinas on P12, after 12 hours in room air following 75% O_2 exposure. These expression patterns are consistent with previous studies (Pierce *et al*., 1995), but a comparable increase in VEGF expression was not seen in any region in *Pdeb*^{rd1}/*Pdeb*^{rd1} mouse retinas after 75% O_2 exposure.

2.2. Regression of established proliferative retinopathy in patient at the onset of clinically detectable retinal degeneration (IV)

As a clinical counterpart to the mouse experiment in which an exogenous stimulus of pathological formation of new retinal blood vessels fails in the presence of advanced photoreceptor cell degeneration, we presented a clinical case in which proliferative retinopathy regressed spontaneously in a diabetic patient with concurrent retinitis pigmentosa.

On fundus examination of a 36-year-old female, diagnosed with type I diabetes mellitus for the past 34 years, we observed in both eyes inactive fibroglial membranes projecting into the vitreous from the optic discs (IV, Fig. 3). This pattern was consistent with regressed retinal neovascularization, often observed in cases of patients with proliferative diabetic retinopathy after successful laser treatment (Roof and Makino, 2000). However, this patient had never received laser treatment. In the periphery and midperiphery of the fundus, attenuated vasculature and atrophic retina with granular and bone spicule pigmentary changes were observed, consistent with a diagnosis of retinitis pigmentosa (IV, Fig. 3) and confirmed by a virtually flat electroretinogram (i.e., less than 10 μV). In non-diabetics with retinitis pigmentosa, spontaneous regression of optic disc neovascularization caused by an unknown mechanism can also occur (Hayakawa *et al*., 1993). This example and other sporadic clinical case reports (Butner, 1984; Uliss *et al*., 1986; Hayakawa *et al*., 1993) suggest that in the clinical setting of rod photoreceptor cell degeneration, proliferative retinopathies may fail to develop or regress early.

3. Development of new strategies in phage display –technology for the study of endothelial cells *in vitro* and *in vivo* (V, VI)

3.1. Probing target cell surfaces by biopanning and rapid analysis of selective interactive ligands (BRASIL; V)

We devised a method termed biopanning and rapid analysis of selective interactive ligands (BRASIL) from an assay described to measure receptor binding of specific yet low-affinity ligands (Hatzfeld *et al*., 1982; Ouaissi *et al*., 1984; Levesque *et al*., 1985; Giordano *et al*., 1994).

We set out to study cell binding by using BRASIL to test an established ligand-receptor pair. Experiments were designed using RGD-4C phage

(displaying the motif ACDCRGDCFCG, termed RGD-4C peptide), which is a specific ligand for α_v integrins (Pasqualini *et al*., 1997). We reasoned that cell-surface–bound phage could be specifically carried through an organic phase and recovered by infection of the host bacteria. α_v integrin-expressing Kaposi's sarcoma cells were detached, incubated on ice with RGD-4C phage or control insertless phage (fd-tet), and the mixture was separated by differential centrifugation through the organic phase. Phage recovery correlated directly with increasing phage input and the recovery ratio (phage output from organic lower layer/phage input from aqueous upper layer), and decreased with host bacteria saturation (V, Fig. 1A). Under non-saturating conditions, the ratio of specific (RGD-4C) phage to control (fd-tet) phage (termed 'enrichment') ranged from 100 to 500. We also showed that the binding of RGD-4C phage to KS1767 cells is specific since such cell-phage binding was inhibited by the cognate synthetic RGD-4C peptide in a dose-dependent manner; while negative control peptides GRGESP (V, Fig. 1B) or CARAC at the same molar concentrations had no inhibitory effect. No phage were present at the bottom of the tube or in the organic phase in the absence of KS1767 cells. Next, we compared BRASIL with conventional cell-panning strategies that require washing steps. The number of RGD-4C phage recovered by BRASIL was significantly higher than using a conventional phage-cell binding strategy involving washing (V, Fig. 1C). Conversely, significantly lower background with the negative control phage was observed (V, Fig. 1C). Given the significant increase in recovery of specific phage and the substantial decrease in background, the overall accuracy (cell-specific phage recovery) improved consistently by more than one order of magnitude when BRASIL was used relative to conventional cell-panning methods.

Next, we tested whether BRASIL could also be used to screen phage display random peptide libraries on cells. We designed a two-step panning strategy to isolate phage that bind to angiogenic (VEGF-stimulated) endothelial cells. First, to decrease non-specific binding, we pre-cleared the phage library on starved endothelial cells (before panning on the same cell line stimulated with recombinant $VEGF_{165}$; starved HUVECs were incubated with the phage library and centrifuged through the organic phase (V, Fig. 2A). Second, the unbound phage pool left in the aqueous phase was transferred to a fresh tube and incubated with VEGF-stimulated HUVECs. After centrifugation through the organic phase, phage bound to the VEGF-stimulated HUVEC pellet were recovered by bacterial infection, amplified and subjected to two more rounds of selection (V, Fig. 2A).

To asses the efficiency of the selection method, we compared 21 random phage clones for binding to starved and VEGF-stimulated HUVECs. Fourteen out of 21 clones (67%) had a greater than 150% enhancement in the ratio of cell binding upon VEGF stimulation normalized to control insertless phage. Sequence alignment analysis of 34 clones randomly chosen from the selected phage revealed that 24 clones (70%) of the phage recovered by BRASIL had peptide motifs that could be mapped to sequences present in VEGF family members (V, Table 1).

3.2.Validation of a chimeric ligand mimic of VEGFR-1 and neuropilin-1 (V)

We chose the CPQPRPLC phage and CNIRRQGC phage (a representative phage out of three different clones displaying the motif IRRE/Q) for *in vitro* phage binding assay on VEGFR-1. The receptor was immobilized on a microtiter well-plate and incubated with CPQPRPLC phage, CNIRRQGC phage or fd-tet as a negative control. Both CPQPRPLC and CNIRRQGC phage bound to VEGFR-1 (V, Fig. 2B). The CPQPRPLC phage bound best with an average of over 1,000-fold enrichment observed over each of the controls used: CPQPRPLC phage binding to VEGFR-1 over bovine serum albumin and CPQPRPLC phage over fd-tet phage binding to VEGFR-1 (V, Fig. 2B).

The CPQPRPLC sequence matched motifs found within the VEGF-B isoforms (V, Fig. 2C). VEGF-B has two mRNA splice variants generated by the use of different, but overlapping, reading frames of exon 6 (isoforms 167 and 186), which diverge in sequence in their C termini (Olofsson *et al*., 1999). The pentapeptide motif PRPLC is found in the recombinant human $VEGF\text{-}B_{167}$ C-terminal region encoded by exon 6B, starting at the second residue after the boundary between exons 5 and 6B. PRPLC is a NRP-1 binding domain (Makinen *et al*., 1999). On the other hand, the tetrapeptide motif PQPR—which overlaps with PRPLC and also with the phage-displayed CPQPRPLC peptide—is found in the C terminal of recombinant human $VEGF\text{-}B_{186}$, and encoded by exon 6A. PQPR is embedded within a 12-residue known NRP-1 binding site (Makinen *et al*., 1999). The motif IRRE/Q also showed homology to VEGF family members (V, Table 1) but it was not studied further here.

We evaluated the binding of phage displaying the peptide CPQPRPLC to a panel of VEGF receptors (V, Fig. 3A). In a manner consistent with the receptor recognition profile of VEGF-B (Olofsson *et al*., 1999), CPQPRPLC bound to VEGFR-1 and to NRP-1, but not to VEGFR-2 or to neuropilin-2. CPQPRPLC phage binding to VEGFR-1 and to NRP-1 was inhibited by pre-incubation with $VEGF_{165}$ (V, Fig. 3B) but not with PDGF-BB. These results are consistent with the fact that $VEGF_{165}$ and VEGF-B isoforms compete for binding to VEGFR-1 (Olofsson *et al*., 1999) and suggest that CPQPRPLC and $VEGF_{165}$ might recognize closely related or overlapping binding sites. Finally, binding of CPQPRPLC phage to the immobilized VEGFR-1 and NRP-1 was specific because it could be inhibited by the corresponding synthetic peptides in a concentration-dependent manner (V, Fig. 3C and D).

These data show that CPQPRPLC is a chimeric VEGF-B-family mimic, and that this peptide interacts specifically with VEGFR-1 and NRP-1.

3.3. Probing human blood vessels with a phage display random peptide library *in vivo* (VI)

We reasoned that *in vivo* selection of phage-display random peptide libraries in humans would advance the identification of human vascular targeting probes and facilitate development of targeted delivery of therapeutic and imaging agents to the vasculature. We took the initial step toward developing an *in vivo* phage display–based, ligand–receptor map of human blood vessels.

A large-scale preparation of a phage random peptide library displaying the insert CX_7C was constructed to create the highest possible insert diversity. The diversity of the library was determined to be $2x10^8$ and its final titer was approximately $1x10^{12}$ TU per ml. A 48-year-old male patient with Waldenström macroglobulinemia, who after massive intracranial bleeding remained comatose with progressive and irreversible loss of brainstem function until the patient met the formal criteria for brain-based determination of death (Wijdicks, 2001), received an intravenous infusion of $1x10^{14}$ TU of the unselected CX_7C phage library. 15 minutes after phage library infusion tissue samples were obtained from bone marrow, prostate, liver, fat-tissue, skeletal muscle and skin, to provide histopathological diagnosis and to recover phage from these organs (VI, Fig. 1A). Tissues were weighted and homogenized, and phage were recovered by bacterial infection. Binding of the phage pool was quantified by calculating the number of recovered phage titers from the recovered tissue samples. Phage recovered from each tissue were processed for sequencing of the peptide inserts, as well as from the unselected library.

To analyze the distribution of inserts from the random peptide library, we designed a high-throughput pattern recognition software to analyze short amino-acid residue sequences. This automated program allowed surveillance of peptide inserts recovered from the phage library screening. Based on SAS (version 8; SAS Institute) and Perl (version 5.0), the program conducts an exhaustive amino-acid residue sequence count and keeps track of the relative frequencies of n distinct tripeptide motifs representing all possible n_3 overlapping tripeptide motifs in both directions ($n << n3$). This analysis was applied for phage recovered from each target tissue and for the unselected CX_7C random phage-display peptide library. Counts were recorded for all overlapping interior tripeptide motifs, subject only to reflection and single-voting restrictions. No peptide was allowed to contribute more than once for a single tripeptide motif (or a reversed tripeptide motif). Tripeptide motifs in both directions are chemically nonsymmetrical and not necessarily equivalent. However, because we often recover forward and reverse tripeptides recognizing the same receptor by *in vivo* phage display, we chose to take this redundancy into account, with the understanding that this is not a general feature that is applicable to every ligand–receptor pair interaction. Each peptide contributed five tripeptide motifs. Tripeptide motif counts were conditioned on the total number for all motifs being held fixed within a tissue. The significance of association of a given allocation of counts was assessed by the one-tailed Fisher's exact test. Results were considered statistically significant at $P < 0.05$. In summary, to test for randomness of distribution, we compared the relative frequencies of a particular tripeptide motif from each target to those of the motifs from the unselected library; such an approach is intrinsically a large-scale contingency table association test.

To determine the distribution of the peptide inserts homing to specific sites after intravenous administration, we compared the relative frequencies of every tripeptide motif from each target tissue to those from the unselected library. We analyzed 4,716 phage inserts recovered from the five tissues and from the unselected library. Tripeptide motifs were chosen for the phage insert analysis because three amino-acid residues seem to provide the minimal framework for structural formation and protein–protein interaction (Vendruscolo *et al.*, 2001). Each phage insert analyzed contained seven amino-acid residues and contributed five potential tripeptide motifs; thus, counting both peptide orientations, a total of 47,160 tripeptide motifs were surveyed.

Comparisons of the motif frequencies in a given organ relative to those frequencies in the unselected library showed a nonrandom nature of the peptide distribution (VI, Table 1); such a bias is particularly noteworthy given that only a single round of *in vivo* screening was performed. Of the tripeptide motifs selected from tissues, some were preferentially recovered in a single site whereas others were recovered from multiple sites. These data are consistent with some peptides homing in a tissue-specific manner and others targeting several tissues. We next adapted the ClustalW software from the European Molecular Biology Laboratory to analyze the original cyclic phage peptide inserts of seven amino-acid residues containing the tripeptide motifs. This analysis revealed four to six amino-acid residue motifs that were shared among multiple peptides isolated from a given organ (VI, Fig. 2). We searched for each of these motifs in online databases (http://www.ncbi.nlm.nih.gov/BLAST through the National Center for Biotechnology Information) and found that some appeared within known human proteins. As our motifs are likely to represent sequences present in circulating ligands (either secreted proteins or surface receptors expressed on circulating cells) that home to vascular receptors, we compiled a panel of candidate human proteins potentially mimicked by selected peptide motifs (VI, Table 2).

3.4. Validation of a candidate ligand-receptor pair in prostate vasculature (VI)

We isolated from the prostate a potential mimetope of interleukin 11 (IL-11), which has been previously shown to interact with receptors within endothelium and prostate epithelium (Mahboubi *et al*., 2000; Campbell *et al*., 2001). Because of the availability of the IL-11 receptor (IL-11R) and well-characterized interaction between the candidate ligand (IL-11) and its receptor, we chose the motif RRAGGS, a peptide mimic of IL-11 (VI, Table 2), for validation. We show by phage overlay on human tissue sections that a prostate-homing phage displaying an IL-11 peptide mimic specifically bound to the endothelium and to the epithelium of normal prostate (VI, Fig. 3A), but not to control organs, such as skin (VI, Fig. 3B). In contrast, a phage selected from the skin (displaying the motif HGGVG), did not bind to prostate tissue (VI, Fig. 3C); however, this phage specifically recognized blood vessels in the skin (VI, Fig. 3D). Moreover, the immunostaining pattern obtained with an antibody against human IL-11R on normal prostate tissue (VI, Fig. 3E) is indistinguishable from that of the CGRRAGGSC-displaying phage overlay (VI, Fig. 3A); a control antibody showed no staining in prostate tissue (VI, Fig. 3F). These findings were recapitulated in multiple tissue sections obtained from several different patients.

Finally, by using a ligand–receptor binding assay *in vitro*, we demonstrate the interaction of the CGRRAGGSC phage with immobilized IL-11R at the protein–protein level. Recombinant IL-11Rα, VEGFR-1, and leptin receptor (OB-R; homologous to a co-receptor of IL-11Rα) were immobilized on microtiter wells and incubated with the CGRRAGGSC phage, an unrelated phage clone (displaying the peptide CRVDFSKGC) or insertless phage (fd-tet). The CGRRAGGSC phage bound to the IL-11Rα in contrast to the control receptors and control phage (VI, Fig. 4A). Such binding was specific because it was inhibited by the native IL-11 ligand, but not a negative control IL-1, in a concentration-dependent manner (VI, Fig. 4B).

Discussion

1 Novel role for CD13/APN and APA as regulators of angiogenesis and vascular receptors for circulating ligands (I-III)

Several approaches indicate that the vascular endothelium of angiogenic blood vessels express surface markers that are accessible from the circulation in tumors but are undetectable, inactive, or inaccessible in normal blood vessels. Such cell markers include growth factor receptors, cell adhesion molecules and proteoglycans (Brooks *et al*., 1994a; Pasqualini *et al*., 1997; Arap *et al*., 1998; Brekken *et al*., 1998; Burg *et al*., 1999). Membrane-associated proteases such as gelatinases have also been found to be molecular targets in tumor blood vessels (Koivunen *et al*., 1999). In the work presented here, we described the identification of two membrane-associated aminopeptidases, CD13/APN and APA, as functional markers of angiogenic blood vessels.

NGR-peptide was previously isolated as a peptide motif homing to tumor vasculature (Arap *et al*., 1998). We showed that tumor-homing NGR peptides bind to CD13/APN. We also showed that CD13/APN is a new marker for angiogenic vasculature, and that it is functionally important in angiogenesis. Soon after discovering the functional role CD13/APN had in angiogenesis, we hypothesized that APA, a closely related membrane aminopeptidase, could also have a functional role in this process. We predicted that genetic elimination of APA or biochemical inhibition of its enzymatic activity would lead to insights into mechanisms of abnormal blood vessel formation. We also reasoned that identification of a specific APA ligand might yield a desirable targeting tool, given the high expression of this protease in the newly formed blood vessels of human tumors (Schlingemann *et al*., 1996).

The phage binding assays and *in vivo* homing experiments in human tumor xenografts in mice showed that NGR peptides bind selectively to CD13/APN. Phage displaying NGR-peptides interacted with immunocaptured CD13/APN and CD13/APN-transfected cells in culture. This binding is specific; in each case, the binding was inhibited by the cognate soluble peptide. Furthermore, anti-APN antibody inhibited *in vivo* homing of NGR phage to human tumor xenografts in mice, strongly suggesting that CD13/APN is the receptor for NGR peptides in the tumor vasculature.

The expression pattern of CD13/APN agrees with its proposed role as the receptor for the NGR peptides in tumor vasculature; CD13/APN is specifically expressed in endothelial and periendothelial cells in angiogenesis. Various types of tumors in two species, analyzed with two monoclonal anti-APN antibodies and with an NGR phage overlay, consistently revealed CD13/APN expression in tumor vasculature. The vascular CD13/APN expression was independent of whether the tumor cells expressed CD13/APN. We also found strong CD13/APN expression in the blood vessels of corpus luteum and have shown in other work that retinal neovasculature expresses CD13/APN. In each case, in tumors, in the corpus luteum, and during retinal neovascularization, the vasculature is undergoing angiogenesis (Smith *et al*., 1994; Folkman, 1995; Hanahan and Folkman, 1996; Klauber *et al*., 1997). Thus, CD13/APN expression correlates with angiogenesis. We did not find CD13/APN expression in the vasculature of any normal human tissues, including the blood vessels in the brain. However, others have found CD13/APN in the pericytes associated with the blood-brain barrier (Kunz *et al*., 1994). Although we are uncertain as to the cause, this discrepancy may relate to expression levels, which appear to be far higher in angiogenesis than in resting blood vessels. Importantly, both studies found the endothelial cells in the brain to lack CD13/APN.

We showed that CD13/APN is not only a previously unrecognized marker of angiogenic endothelial cells but is also functionally important in angiogenesis since CD13/APN inhibitors, such as inhibitory antibodies R3-63 and 2M-7, and chemical inhibitors bestatin and actinonin, were found to suppress angiogenesis. In accordance with earlier studies (Fujii *et al*., 1995; Saiki *et al*., 1993) the CD13/APN inhibitors also suppressed tumor growth. Our results suggest that inhibition of angiogenesis is likely to be a factor in the anti-tumor activity of these compounds. The role of CD13/APN in angiogenesis may be to facilitate endothelial cell invasion of tissue, which is an essential component of angiogenesis (Bussolino *et al*., 1997; Zetter, 1998). Experiments with tumor cells have shown that CD13/APN expression can increase cell invasiveness (Fujii *et al*., 1995). Because compounds that are enzymatic inhibitors of CD13/APN inhibit angiogenesis, this putative invasion-promoting effect would appear to be related to the enzymatic activity of CD13/APN. Another possibility is that CD13/APN could modulate the activity of growth factors or cytokines. Such activities are common among aminopeptidases, including CD13/APN (Ward *et al*., 1990; Taylor, 1993; van Hal *et al*., 1994; Lendeckel *et al*., 1999).

The expression of CD13/APN in angiogenesis may depend on growth factors and cytokines; TNF-α and bFGF have previously been shown to up-regulate CD13/APN expression in cells (Riemann *et al*., 1995; van Hal *et al*., 1994). Consistent with the role of CD13/APN as an angiogenic regulator in vascular endothelial cells, we showed that hypoxia markedly induces endogenous *CD13/APN* mRNA and reporter gene transcription levels in endothelial cells. During the initial stages of angiogenesis, signals generated by hypoxic stimuli alter the expression of many genes contributing to angiogenic differentiation, including those encoding glycolytic enzymes, glucose transporters, and angiogenic and hematopoietic growth factors (Semenza, 1999). Expression of these genes can be affected either

positively or negatively through transcriptional and posttranscriptional mechanisms (Semenza and Wang, 1992; Ikeda *et al*., 1995; Schmedtje *et al*., 1997; Discher *et al*., 1998; Kroll *et al*., 1999). The elevation of *CD13/APN* expression could be a direct response to hypoxia-activated DNA-binding transcription factors, such as HIF-1 or hypoxia-associated factor, or a secondary consequence of hypoxia-induced autocrine growth factor production. Examination of the sequence of the *CD13/APN* proximal promoter failed to reveal a consensus HIF-1 (Wang and Semenza, 1993) or EP-17 (Gupta *et al*., 2000) binding site; however, numerous other consensus sites for transcription factors important for angiogenesis are present. Identification of the specific transcription factors regulating *CD13/APN* gene expression in response to angiogenic stimuli will be important for elucidating the molecular mechanisms controlling its angiogenic activation.

We also tested a panel of cytokines whose expression has been shown to be up-regulated by hypoxia. Of those specific cytokines tested, *CD13/APN* mRNA, protein, and promoter activity levels were increased most strikingly in response to bFGF and VEGF. These factors are co-expressed in a variety of cancers and functionally complement each other during angiogenesis (Hanahan and Folkman, 1996; Koolwijk *et al*., 1996). Thus, it is reasonable to assume that they would play a similar role in the angiogenic induction of *CD13/APN*. The regulation of specific genes by bFGF and VEGF has been shown to initiate signaling cascades involving several different intermediates including Ras, phospholipase C, p125FAK, and PI3K (reviewed in Tallquist *et al*., 1999). Finally, functional antagonists of CD13/APN interfere with tube formation but not proliferation of primary vascular endothelial cells, suggesting that CD13/APN functions in the control of endothelial cell morphogenesis. These studies clearly establish the CD13/APN aminopeptidase as an important regulator of endothelial morphogenesis during angiogenesis. Indeed, following the studies described in this thesis, Bhagwat *et al.* showed that *CD13/APN* is a transcriptional target of Ras/MAPK and PI3K pathways, two Ras-mediated signaling pathways that have been implicated to play a role during the angiogenic switch. Furthermore, under conditions where CD13/APN up-regulation is prevented by inhibiting the Ras/MAPK and PI3K pathways, exogenous expression of CD13/APN can rescue the angiogenic progression in an *in vivo* model of angiogenesis (Bhagwat *et al*., 2003).

Our first insights to the functional role for APA in angiogenesis derived from the *in vivo* studies with APA knockout mice. Knockout APA -/- mice develop normally (Lin *et al*., 1998), but our study shows a severely impaired angiogenic response in oxygen-induced retinopathy in a mouse model of retinopathy of prematurity (Smith *et al*., 1994) as well as in an *in vivo* model of angiogenesis where angiogenic growth factors are used to induce new blood vessel formation into a subcutaneous gelfoam plug (McCarty *et al*., 2002). The fact that APA -/- mice develop normally with no gross phenotypic abnormalities suggests that while APA may participate in embryonic angiogenesis, it is not an essential participant. The molecular events associated with *de novo* formation of blood vessels during early development may be redundant or compensated by other proteases. Our data indicate a functional role for APA in the pathological formation of new blood vessels from a preformed vascular bed. These data strengthen the candidacy of APA as a specific vascular target to inhibit abnormal angiogenesis associated with tumor formation and retinal neovascularization.

In order to obtain molecular probes for studying the role of APA activity in angiogenesis as well as ligands to target tumor blood vessels *in vivo,* we screened a combinatorial peptide library for APA binding peptides. We selected APA-binding phage displaying the sequences CYNLCIRECESICGADGACWTWCADGCSRSC and CPKVCPRECESNC that bound to APA. Their binding to APA was specifically inhibited by the synthetic peptide with a consensus APA binding motif CPRECESIC. Binding of one of the isolated phage (CLGQCASICVNDC) to APA was not inhibited by CPRECESIC peptide, possibly due to the short sequence shared with the other isolated peptides leading to a low-affinity interaction suggesting, that the sequence CPRECES alone is sufficient to promote inhibition. In addition to binding APA *in vitro*, the CPKVCPRECESNC phage also homed to the vasculature of human tumor xenografts and murine tumors *in vivo* indicating that APA binding peptides can be used as ligands to target APA in tumor vasculature for the purpose of systemic delivery of therapeutics. Since a search of human sequence databases for sequences homologous to the selected peptides did not yield matches consistent with potential endogenous APA-ligands, the biological substrate responsible for APA inhibition by the consensus motif CPRECESIC may remain unknown until three-dimensional structure of the interaction of ligand peptides and the APA active site has been solved.

In vitro and *in vivo* angiogenesis assays were performed to further define the functional basis for the role of APA in blood vessel formation. Because our evaluation of APA enzyme activity in freshly isolated endothelial cells led to the observation of high enzyme activity in HMECs and in some tumor-derived endothelial cells, we used HMECs in our *in vitro* angiogenesis assays. We found that the synthetic CPRECESIC peptide inhibited VEGF-induced migration and proliferation of HMECs. CPRECESIC peptide also inhibits cord/ tube formation of microvascular endothelial cells in a Matrigel assay and angiogenesis in VEGF-stimulated CAMs.

In addition to mouse models, we evaluated whether abnormal angiogenesis associated with certain human diseases is related to the expression of APA in the vasculature. We examined the expression and activity of APA in malignant

gliomas and metastatic carcinomas to the brain and showed that APA is not only present in angiogenic blood vessels of human malignant gliomas but also that the protein overexpressed in the perivascular cells is enzymatically active. In contrast, the presence of the enzyme and its corresponding proteolytic activity were undetectable in the blood vessels and adjacent normal brain. Pericytes, which were once seen merely as the contractile microvessel equivalent of smooth muscle cells surrounding larger blood vessels, also play an active role in neovascularization and maturation, remodeling and maintenance of the vascular system (Schlingemann *et al*., 1990; Schlingemann *et al*., 1991; Allt and Lawrenson, 2001; Morikawa *et al*., 2002). Similarly to endothelial cells, perivascular cells also exhibit molecular, functional and structural heterogeneity (Morikawa *et al*., 2002), of which overexpression of APA, as well as CD13/APN, in tumor blood vessels are good examples. APA may play a significant role in several functions of perivascular cells, such as secretion of growth factors, modulation of the ECM and regulation of vascular permeability.

Overexpression of APA in activated blood vessels has been associated with perivascular cells in human tumors here and elsewhere (Schlingemann *et al*., 1996) but the exact cellular location of active APA in the vascular endothelium of blood vessels during tumorigenesis is still not entirely clear. A further level of complexity arises because tumor cells per se express APA in several human cancers (Finstad *et al*., 1985; Nanus *et al*., 1998; Fujimura *et al*., 2000; Ino *et al*., 2000) making it difficult to differentiate the origin of APA enzymatic activity. In the study presented here, we showed that APA expression and activity are strongly increased in tumor blood vessels during late stages of human malignant glioma progression. We have also observed, that endothelial cells derived from mature macrovessels like human umbilical vein endothelial cells, express little or no APA, while HMECs, extracted from small, immature blood vessels show much higher APA expression. One might speculate that this process mimics an angiogenic switch (Hanahan and Folkman, 1996) in which endothelial cells are recruited to form new capillaries for extensive tumor growth. Once tumor blood vessels mature to the stage at which they are covered with perivascular cells, APA expression is reduced in the endothelial cells. In turn, activated perivascular cells covering more mature tumor blood vessels express high levels of APA.

Given that the selected APA-binding phage home to tumor vasculature, and the consensus motif deduced from APA-binding peptides specifically inhibit APA enzymatic activity, we evaluated whether APA-binding peptides can be used as targeted inhibitory carriers. Although APA is also expressed in many cell types in normal tissues (Li *et al*., 1993b; Schlingemann *et al*., 1996; Alliot *et al*., 1999), our results showed that APA is exposed, active, and available for binding to circulating ligands from the luminal side of the vascular endothelium because APA-binding phage selectively targets tumor vasculature in human breast carcinoma-derived tumor xenografts. After documenting the expression of the target in the tumor vasculature, in a mouse model of mammary carcinoma (Deroanne *et al*., 1997; Hajitou *et al*., 2001), we successfully targeted this tumor with the APA-binding phage and chose this model for therapy experiments. We showed that APA-inhibitory ligands--such as the described APA-binding peptides or anti-APA monoclonal antibodies (Assmann *et al*., 1992) --specifically target angiogenic vasculature and suppress tumor growth *in vivo* in this experimental system. The IC_{50} of the ASD-37 monoclonal antibody for the APA enzyme activity was found to be ~60 nM. The apparently superior inhibitory ability of anti-APA monoclonal antibodies is commensurate with and one likely explanation for the more evident histopathological findings observed after treatment with such antibodies relative to our APA-binding peptides. Considering these promising pre-clinical results, and the fact that antibody-related toxicity occurred only when mice received over 20-fold the doses required to produce the anti-tumor activity in the present study (Assmann *et al*., 1992; Mentzel *et al*., 1999), anti-APA inhibitory antibodies should be at least considered as candidates for drug development and, possibly, translation into clinical applications.

How CD13/APN and APA facilitate angiogenesis is still not known. The function of CD13/APN appears to depend on the availability of its substrates, thus the location the enzyme; its location on the cell surface mandates that its functional activity is dictated by substrates that are available in the immediate intercellular space. CD13/APN has been implicated in the catabolism of neuroactive peptides (Matsas *et al*., 1984; Konkoy *et al*., 1996; Noble and Roques, 1997), amino acid scavenging and degradation of regulatory peptides (Turner *et al*., 1987; Rawlings and Barrett, 1993), cell adhesion alterations (Menrad *et al*., 1993), tumor invasion and metastasis (Saiki *et al*., 1993; Fujii *et al*., 1995;), as well as antigen processing and presentation (Mouritsen *et al*., 1992; Falk *et al*., 1994). Because the switch from the quiescent to angiogenic endothelial phenotype involves an alteration in the relative levels of angiogenic inhibitors and activators (Hanahan and Folkman, 1996), it is intriguing to postulate a role for CD13/APN in the processing of small regulatory molecules required to initiate, maintain, or suppress the angiogenic program in tumor vessel endothelium. CD13/APN is also present in human plasma as a soluble form. Soluble CD13/APN activity is elevated in cancer patient plasma and effusions and this activity has a strong correlation with the tumor load suggesting that the soluble plasma CD13/APN is, at least, partly originating from tumor/ tumor endothelium (van Hensbergen *et al*., 2002). Interestingly, CD13/APN has recently been suggested as a prognostic marker for pancreatic carcinoma patients and the

CD13/APN gene expression in pancreatic carcinoma tumors was associated with an increase of the intratumor microvessel density (Ikeda *et al.*, 2003). An intriguing feature of CD13/APN is that its expression and enzymatic activity can be physiologically regulated. Its activity and substrate specificity depend on conformational changes induced by various stimuli, including proliferative signals. Studies using monoclonal antibodies indicate that CD13/APN undergoes regulatory intramolecular alterations that result in exposure of cryptic sites and regulation of enzyme activity (Xu *et al.*, 1997). The immunoreactivity patterns obtained with cultured cells and tissue sections from kidney, breast, and prostate carcinomas suggest that different CD13/APN forms are expressed in myeloid cells, epithelia, and tumor-associated blood vessels (Curnis *et al.*, 2002). Association with proteins or factors present only in the tumor microenvironment might cause differential reactivity or accessibility to different CD13/APN ligands, such as the tumor vasculature targeting NGR-peptide ligand.

Even less is known about the natural substrates and function of APA. It also appears to be a molecule that has different functions, according to the organ and time period examined. A broad spectrum of tissues expresses APA (Li *et al.*, 1993b), but its only well understood role is in the conversion of AngII to AngIII in the renin-angiotensin system (Jackson, 2001). *In vivo*, AngII remains the best-characterized substrate for APA.

Interestingly, angiogenesis regulators that belong to the renin-angiotensin system are degraded by APA and CD13/APN, suggesting a synergistic role of these enzymes in the generation of pro-angiogenic molecules such as AngIII and AngIV (Stroth and Unger, 1999). In the renin-angiotensin system, renin converts angiotensinogen to AngI and ACE converts AngI into AngII, which seems to be the major effector in blood pressure and homeostasis control. APA further converts AngII into AngIII (Jackson, 2001). While angiotensins are usually investigated in relation to mechanisms of arterial hypertension, several studies suggest a role for angiotensins in blood vessel formation (Andrade *et al.*, 1996; le Noble *et al.*, 1996; Munzenmaier and Greene, 1996; Walsh *et al.*, 1997; Monton *et al.*, 1998;Nadal *et al.*, 2002). Furthermore, inhibition of ACE suppresses angiogenesis (Volpert *et al.*, 1996; Yoshiji *et al.*, 2001). However, involvement of ACE in vessel formation is not so simple, since physiological angiogenesis seems to be improved by the use of ACE inhibitors (Takeshita *et al.*, 2001; Emanueli *et al.*, 2002). In another study AngII was suggested to play a role as a humoral regulator of peripheral angiogenesis involving two receptor subtypes with opposing actions. AngII exhibited an anti-angiogenic effect through the AT1 receptor and pro-angiogenic effect through the AT2 receptor (Walther *et al.*, 2003). The established involvement of angiotensins in angiogenesis referenced above and the overexpression of APA and CD13/APN in angiogenic blood vessels suggest a mechanism for these peptidases in neovascularization.

As predicted by our hypothesis that APA is a functional target for molecular agents that can inhibit angiogenesis, we observed a decreased neovascularization in APA -/- mice following induction of angiogenesis by hypoxia and by growth factors and reduced tumor growth in mice treated with APA inhibitors. We have also demonstrated that CD13/APN has a functional role in angiogenesis. Our data are consistent with those showing a stimulatory effect of angiotensins on endothelial cell migration (Kifor and Dzau, 1987) and proliferation (Monton *et al.*, 1998). Also, angiotensin receptors are expressed in the CAM, suggesting that angiotensins might play a role in angiogenesis and capillary branching (le Noble *et al.*, 1996). Our results, however, could also reflect a more general effect of APA and CD13/APN on angiogenic mechanisms such as a role in degradation of ECM by processing other unknown substrates (Sang, 1998). Sequential degradation of ECM proteins or other angiogenic proteins may generate bioactive peptides that are able to regulate angiogenesis either positively or negatively. Natural and synthetic inhibitors of other metalloproteases inhibit not only *in vivo* angiogenesis (Sang, 1998; Koivunen *et al.*, 1999; Egeblad and Werb, 2002) but also migration and proliferation of microvascular cells *in vitro* (Murphy *et al.*, 1993; Zempo *et al.*, 1996). Furthermore, knockout mice in which other metalloproteases have been eliminated (such as MMP-2 or -9) show a defect in angiogenesis (reviewed in Egeblad and Werb, 2002).

Due to their specific expression and accessibility on tumor blood vessels APA and CD13/APN in the cell membranes of activated endothelial cells or perivascular cells can be systemically targeted, making them suitable candidate receptor for targeted imaging or therapy. Openings between defective endothelial cells of tumor blood vessels (Hashizume *et al.*, 2000) enable access to the perivascular cell layer of the vasculature; other cell surface receptors of activated perivascular cells (such as the proteoglycan NG2) have been targeted by *in vivo* phage display (Burg *et al.*, 1999). Combinatorial strategies targeting both the endothelial and perivascular cell compartments are likely to provide improved efficacy for antiangiogenic therapies in multiple stages of tumorigenesis (Bergers *et al.*, 2003). Following the identification of the CD13/APN peptide ligand homing to angiogenic vasculature, it has been successfully used to target an array of therapies to tumor vasculature including cytotoxic drugs (Arap *et al.*, 1998), pro-apoptotic peptides (Ellerby *et al.*, 1999), cytokines (Curnis *et al.*, 2000) and liposomes (Pastorino *et al.*, 2003) showing marked therapeutic efficacy in tumor-bearing mouse models.

In summary, our results have revealed new roles for APA and CD13/ APN as functional targets in angiogenic vasculature that may contribute to an important regulatory pro-angiogenic pathway.

These peptidases are expressed in both the endothelial and periendothelial cell compartment of angiogenic blood vessels and are therefore accessible to circulating ligands and may therefore be used to target therapies to angiogenic vasculature. Their enzyme activities regulate the angiogenesis process, since either genetic or biochemical ablation of the activity of these enzymes significantly reduces the formation of new blood vessels in several pathological states, such as cancer and retinopathies. The APA and CD13/APN ligand motifs described here may therefore serve as a peptidomimetic drug leads against angiogenic mechanisms. Taken together, the studies described in here showed that APA and CD13/APN are specific vascular targets to be critically evaluated in translational clinical trials against pathologic angiogenesis.

2. Absence of retinal angiogenesis under reduced metabolic stress (VI)

Angiogenesis is a hallmark of eye diseases ranging from retinopathy of prematurity in oxygen-treated neonates to diabetic retinopathy in adults to age-related macular degeneration in the elderly. We asked what local factors might account for the reason why the retina is especially or uniquely damaged by pathological neovascularization in diabetic retinopathy and retinopathy of prematurity. A feature distinguishing the retina from other parts of the central nervous system and from other organs is the presence of large numbers of specialized photoreceptor cells—140 million rod photoreceptor cells and 6 million cone photoreceptor cells. We hypothesized that these retinal diseases may be based on the unusual signaling properties of photoreceptor cells (Pugh *et al.*, 1999; Ebrey and Koutalos, 2001; Hurley, 2002; Hammes *et al.*, 2003) and on the continual proximal growth and distal shedding of their outer segments throughout life (Young and Bok, 1969). These hypotheses lead to strong predictions about epidemiology and treatment, some of which are currently being tested.

Diabetes mellitus is the main cause of a devastating number of new cases of adult blindness and nearly all people with type 1 diabetes show some symptoms of diabetic retinopathy, usually after about 20 years of living with diabetes. Among the many features of this disease, diabetic retinopathy stands out, with its dramatic formation of new blood vessels in the retina, a pathological feature considered to be a hallmark of ischemic retinopathies in general. In the most advanced form of diabetic retinopathy, proliferative diabetic retinopathy, new blood vessels grow uncontrollably on the retinal inner surface, causing hemorrhages and even retinal detachment. Surgical and laser photo-coagulation treatments are only partially effective and may further damage the retinal tissue. Even though well-maintained metabolic control in diabetic patients may decrease the risk of proliferative diabetic retinopathy, new ways for its prevention and treatment are clearly needed. In the retina but not elsewhere, clinical and experimental diabetes cause loss of pericytes, and swelling and damage to capillary endothelial cells that result in the microaneurysms, capillary dropout, leakage, cellular damage and new blood vessel growth that characterize diabetic retinopathy (Kern and Engerman, 1996; Witmer *et al.*, 2003). This microvasculopathy indicates that local factors unique to retina provoke diabetic retinopathy --an unexpected inference about a tissue often considered "an approachable part of the brain" (Dowling, 1987).

A major difference between retina and other central nervous system regions is the photoreceptors. Their signal transduction mechanism is unusually energy-demanding; rods, under conditions of dark adaptation, produce more heat and consume more oxygen than any other cell type. This explains why dark adaptation is disturbed, in a variety of pathological conditions, before other visual functions fail, ranging from *polycythaemia vera* to partial carotid occlusion (Havelius *et al.*, 2000). In normal persons, the sensitivity of rod vision begins to drop when there is a slight reduction of inspired oxygen, equivalent to ascending to 3,000 feet or 1 kilometer (McFarland and Evans, 1939). Thus, the loss of dark adaptation in diabetics with stage 0 retinopathy is understandable, and the much greater loss of rod function during more advanced stages of diabetic retinopathy is well attested. Recordings with oxygen microelectrodes in normal eyes show a precipitous drop in pO_2 as the microelectrode passes inward from the level of the choroidal blood vessels to a minimum in the vicinity of the photoreceptor cell bodies and synapses (Linsenmeier, 1986). In dark-adapted eyes this minimum pO_2 tension is zero, but during an even brief flash of light it reaches 30 mm Hg. Unlike other brain cells, rods function in ultra-low oxygen environments but their intense activity in darkness reduces the pO_2 of the avascular inner retina.

Arden proposed the intriguing hypothesis that the high oxygen consumption of dark-adapted rod cells is the driving force of inner retinal hypoxia, with subsequent VEGF production leading to retinal neovascularization in ischemic retinopathies (Arden, 2001). Hyperglycemia, but especially hypoxia, increases the production of VEGF (Shweiki *et al.*, 1992; Semenza, 2003a). Mild reductions in oxygen delivery in diabetes mellitus are due to many causes and may be important particularly in the retina where the safety margin is so low (Arden, 2001). Arden's hypothesis was indirectly supported by the observation that diabetic retinopathy rarely occurs in retinitis pigmentosa patients (Pruett, 1983; Butner, 1984; Uliss *et al.*, 1986; Hayakawa *et al.*, 1993; Arden, 2001). Anecdotal reports also describe diabetic individuals with absence of diabetic retinopathy in eyes with large retinal scars, although the fellow eye is typically affected (Arden, 2001). In adults with the mitochondrial disorder MIDD 3243, diabetes is characteristic, and diabetic retinopathy commonly occurs unless

retinal degeneration develops (Smith *et al.*, 1999b). Perhaps the best evidence of the importance of hypoxia is the successful treatment of diabetic retinopathy by pan-retinal photocoagulation (Arden, 2001), which may work simply by destroying enough rods to increase retinal pO_2 (Stefansson *et al.*, 1986). Our findings show for the first time that degeneration of rod cells leads to a total lack of reactive retinal neovascularization, accompanied by a failure in the expected VEGF up-regulation. Taken together, these observations from *Pdeb*^{rd1}/ *Pdeb*^{rd1} mice and a human patient afflicted with both diabetes mellitus and retinitis pigmentosa provide direct experimental and mechanistic evidence in support of Arden's hypothesis (Arden, 2001) and suggest that VEGF is a primary link between rod cell numbers and retinal neovascularization. Indeed, it is tempting to speculate that reducing the metabolic rate of rod cells at critical time windows may improve the incidence of retinopathy of prematurity or perhaps slow the progression of diabetic retinopathy in adults. Despite advances in pediatric care, retinopathy of prematurity remains a serious clinical problem. Premature infants are customarily maintained in intensive care units, which are well illuminated, though ordinary care units typically maintain reduced illumination. Extrapolating from the observations of *Pdeb*^{rd1}/ *Pdeb*^{rd1} mice, this is not a correct arrangement. When neonates are in a high-oxygen environment, they should be exposed to as little light as possible. Red light (wavelength >660 nm) is scarcely absorbed by rods; general and local illumination with light emitting diodes should suffice for all necessary manipulations. When the neonate is returned to a normal air environment, the room should be brightly lit, so that the oxygen demand of the still-immature eye is minimized. It is conceivable that increased exposure of premature neonates to light may reduce O_2 consumption by rod photoreceptor cells and retinal hypoxia, ultimately improving their retinopathy. Paradoxically, such reasoning challenges the current recommendation to decrease ambient light exposure in that setting, which has actually failed to prevent retinopathy of prematurity (Reynolds *et al.*, 1998).

It is noted that VEGF is not the only angiogenic mediator whose production in the eye is affected by changes in pO_2 (Ogata *et al.*, 1997; Smith *et al.*, 1997; Khaliq *et al.*, 1998; Yoshida *et al.*, 1998; Dawson *et al.*, 1999; Carmeliet *et al.*, 2001; Ogata *et al.*, 2002). Moreover, VEGF inhibitors and blockers can only partially halt angiogenesis in the retinopathy of prematurity model (Aiello *et al.*, 1995) and not all of patients with diabetic retinopathy show a rise in VEGF (Aiello *et al.*, 1994). Thus, the total absence of retinal neovascularization in homozygous *Pdeb*^{rd1}/ *Pdeb*^{rd1} mice argues that the degeneration of photoreceptor cells may have further effects on angiogenesis that are not VEGF-mediated. Our data do not rule out a possible role for other angiogenic factors known to be regulated by hypoxia, such as TGF-β (Ogata *et al.*, 1997), insulin-like growth factor-1 (Smith *et al.*, 1999a), PlGF (Khaliq *et al.*, 1998) and IL-8 (Yoshida *et al.*, 1998), and more studies are needed to clarify these multiple interactions in the retina.

Growth factors and inhibitors may be involved in coordinating neural and vascular components of the retina by functioning simultaneously as photoreceptor cell survival factors and endothelial cell regulators. bFGF is elevated in *Pdeb*^{rd1}/ *Pdeb*^{rd1} mice several days before photoreceptor cell death (Gao and Hollyfield, 1995) and intra-vitreous injection of bFGF delays the onset of photoreceptor cell degeneration in selected animal models (Faktorovich *et al.*, 1990). PEDF, which is encoded by a gene closely linked to the *Pdeb* locus (Tombran-Tink *et al.*, 1994), is a survival factor for photoreceptor cells (Cayouette *et al.*, 1999) and has been proposed also to play an anti-angiogenic role in the retina (Dawson *et al.*, 1999). Given that PEDF concentration is highest in the matrix surrounding the photoreceptor cell layer (Becerra, 1997; Dawson *et al.*, 1999) that undergoes apoptosis in *Pdeb*^{rd1}/ *Pdeb*^{rd1} mice (Chang *et al.*, 1993; Portera-Cailliau *et al.*, 1994), one might expect that a loss of PEDF would be correlated with an increase rather than a decrease in retinal angiogenesis. In our system, preliminary data based on immunostaining failed to show a correlation between PEDF and neovascularization and suggest that PEDF does not play a major role in the phenomenon described here. However, it is possible that PEDF or other angiogenesis inhibitors are released during the photoreceptor cell apoptotic process, which may contribute to the lack of retinal neovascularization. Roles for additional factors are suggested by the dramatic but unexplained variations in timing of retinal capillary growth into the pigment epithelial cell layer among mutant mice that share a similarity in timing of photoreceptor cell degeneration (Nishikawa and LaVail, 1998). Finally, the neurotransmitter dopamine has been recently shown to inhibit VEGF-induced angiogenesis (Basu *et al.*, 2001). However, dopamine synthesis and utilization are known to be suppressed at least in some mouse models of retinal degeneration (Nir *et al.*, 2000).

In summary, we have shown that ischemia-induced neovascularization of the retina is abolished in a mouse strain with inherited photoreceptor cell degeneration. We also documented that regression of established reactive retinal neovascularization caused by diabetes mellitus could occur in a subset of adult patients also afflicted with retinitis pigmentosa. This striking, previously unreported failure to mount a reactive retinal neovascularization response to potent exogenous stimuli was associated with an absence of the expected VEGF up-regulation in the retina. Our findings support the hypothesis that O_2 consumption by rod cells is a major driving force in ischemic retinal neovascularization and controls VEGF production, although additional trophic agents and cytokines are likely also to be involved in this complex biological phenomenon. Further characterization of this anti-angiogenic state in the

retina may lead to therapeutic approaches against debilitating eye diseases such as ischemic retinopathies and late complications of retinitis pigmentosa.

3. Potential of probing blood vessels by phage display technology (V, VI)

Identification of the endothelial cell surface receptor fingerprint is required for the development of vascular-targeted therapies. Several features in the biology of cell surface receptors point to the rationality of receptor ligand identification for intact receptor molecules embedded in cell membranes instead of isolated receptors. As opposed to purified receptors, membrane-bound proteins are more likely to preserve their active conformation, which are often lost once proteins are removed from their natural environment on the cell membrane. In addition, many cell surface receptors are active as homodimers or heterodimers whose formation may require the cell membrane environment; these interactions further contribute to the ligand specificities of some receptors. Combinatorial approaches for probing the molecular heterogeneity of cell surfaces allow the identification of cell membrane ligands in an unbiased functional assay and without any predetermined notions of the cell surface receptor repertoire; thus, unknown receptors can be targeted. Nonetheless, the great complexity of cell surface molecules still presents a challenge for the isolation of highly specific ligands for a given cell population.

We have developed a new approach for the isolation of cell surface-binding peptides from phage libraries. To circumvent some of the practical difficulties in probing the cell surface, i.e. the recovery of non-specific clones and the loss of cells and subsequent specific phage clones, we developed the BRASIL method for probing the cell surface with phage display libraries. BRASIL method is based on differential centrifugation in which cells of interest incubated with a phage library in an aqueous upper phase are centrifuged through an organic phase separating the unbound phage from the specific phage-cell complex. On side-by-side comparison to current protocols, BRASIL was more sensitive and more specific than techniques that rely on washing or limiting dilution steps to eliminate background during successive rounds of selection. BRASIL method is an efficient and convenient technique for the selection of phage that binds specifically to a given cell surface. We have recently performed a screening of the NCI 60-cell panel using the BRASIL method to create a ligand "fingerprint" for the NCI 60-cell panel demonstrating the applicability of the BRASIL method for high-throughput analysis (Bover *et al.*, *in progress)*. Furthermore, BRASIL method is also more sensitive and more specific than phage selection techniques that rely on washing of the cells, and therefore represents a significant improvement over conventional cell-panning methods. The use of BRASIL method is not limited to random peptide libraries or mammalian cells, but can be used to screen antibody fragment displaying phage libraries and as well as other cell types, such as aspergillosis causing fungus *Aspergillus fumigatus* (J.L., *unpublished observations*).

Since our long-standing interest has been in targeting the vascular endothelium, we screened a phage-display random peptide library on activated, $VEGF_{165}$-stimulated endothelial cells after a library subtraction step with quiescent endothelial cells. We subsequently isolated a CPQPRPLC peptide ligand for VEGF-receptor. This VEGF-receptor ligand appears to be a chimera between overlapping binding sites on different VEGF-B isoforms, since part of it resembles a neuropilin-1 binding site found in VEGF-B_{167} (PRPLC) and the overlapping motif PQPR resembles a neuropilin-1-binding epitope of VEGF-B_{186} (Makinen *et al.*, 1999). Our chimeric peptide ligand interacts specifically with VEGF receptors in a pattern consistent with VEGF-B-type ligands (Olofsson *et al.*, 1999) as confirmed by binding assays with individual phage on a panel of purified targets. We further examined the ability of the synthetic VEGF-receptor ligand to block phage binding to VEGF receptors *in vitro* and found that the isolated peptide ligand is about 100-fold more efficient in blocking phage binding to VEGFR-1 than to neuropilin-1. Because of the observed differential interaction of the chimeric peptide ligand with its' receptors, it is tempting to speculate that our chimeric motif interacts with VEGF receptors differentially. If so, this differential binding may be due to differences in the number of peptide-binding sites on each receptor, or in the affinity of the interaction at each binding site. Alternatively, such ligand-receptor interactions may be dependent on the conditions used for the binding assay. Full understanding of binding mechanisms awaits elucidation of the X-ray crystal structures of VEGFR-1– or NRP-1–CPQPRPLC peptide complexes. Although one cannot as yet assert that BRASIL will be well suited for any cell-selection application, the isolation and further elucidation of vascular receptor-ligand interaction attests to the belief that vascular targets can be found on endothelial cell membranes *in vitro* by using the BRASIL method.

Important application for the BRASIL method can be foreseen in both targeting and identification of ligand-receptor pairs in cell populations derived from patient samples. The method may be used in tandem with fluorescence-activated cell sorting of leukemic cells obtained from bone marrow aspirates from patients or even with circulating endothelial progenitor cells from periferal blood. Tumor- and inflammatory cells from ascites or fine-needle aspirates of solid tumors also seem like ideal clinical material for the identification of novel tumor antigens with the BRASIL method. Moreover, because multiple samples and several selection rounds can be performed in a short amount of time, automation for high-throughput clinical applications is likely to follow.

Besides probing endothelial cell surfaces *in vitro*, the work presented in this thesis describes the identification of vascular receptors and the isolation their peptide ligands by screening blood vessels *in vivo* using phage displayed random peptide libraries. Probing cell surfaces of the blood vessel components *in vivo* may yield to selection of targeting peptides more suitable to clinical applications since they bind to native receptors as they are expressed *in vivo*.

The work done to discover addresses present only in tumor blood vessels but not in blood vessels of normal tissues has been focusing in the use of animal models of human and murine cancers. Based on the work presented in here and previous work by others (Arap *et al.*, 1998; Curnis *et al.*, 2002), we have found it possible to identify vascular receptors in tumor-bearing mice that are also present in human tumors, thus, validating the use animal models to find molecular markers of human blood vessels. However, it is not kown whether targeted delivery will always be achieved in humans by using mouse-derived probes. Extrapolation of the results from mouse experiments to human biology requires that the molecules of interest be expressed and regulated similarly in both species. The prominent species-specific differences in protein expression patterns and ligand–receptor accessibility prompt us to carefully evaluate the information obtained from animal studies before directly applying it to clinical studies. Clearly, the construction of a human vascular map will be of essence in the successful translation of vascular targeting into clinical practice.

The molecular diversity of receptors in human blood vessels remains largely unexplored despite major progress brought about by the human genome project (Lander *et al.*, 2001; Venter *et al.*, 2001). The human genome project has produced a very useful index of genes, but our understanding where and when given genes are expressed lags far behind. Aside from *in vivo* phage display, the use of methods such as SAGE clearly shows that the genetic progression of malignant cells is paralleled by epigenetic changes in nonmalignant endothelial cells induced by angiogenesis of the tumor vasculature (St Croix *et al.*, 2000). Because SAGE is based on differential expression levels of transcripts, it fails to address functional interactions (for example, binding) at the protein–protein level. Potential vascular targets may also be overlooked in high-throughput DNA sequencing or in gene arrays since many of the targets may be expressed in very restricted but highly specific locations in the vascular endothelium and in general, genetic approaches do not take into account the anatomical and functional context of the target molecules. *In vivo* phage display technology has expanded our knowledge of the spatial and temporal expression of vascular markers, and allowed us to take the first steps in elucidating the endothelium based protein-protein interaction map *in vivo*, a field that can be defined as "vascular proteomics" (see Trepel *et al.*, 2002). The complexity of the human endothelium is also apparent from recent studies showing that the profile of certain endothelial cell receptors can vary depending on ethnic background (Wu *et al.*, 2001). In fact, *in vivo* phage-display in humans might reveal diversity of receptors expressed in the blood vessels even at the level of individual patients. However, our validation studies show that at least some ligand–receptor pairs are detectable in multiple unrelated subjects. Another advantage of the method described here is that selected targeting peptides bind to native receptors as they are expressed *in vivo*. Even if a ligand–receptor interaction is mediated through a conformational rather than a linear epitope, it is possible to select binders in the screening.

We started the process towards the construction of the human molecular vascular map by screening a phage displayed random CX_7C peptide library in a patient with Waldenström macroglobulinemia. Recovered peptide inserts, as well as the unselected phage library, were analyzed for the presence of short amino-acid sequences with a high-throughput pattern recognition software comparing the relative frequencies of every tripeptide motif from each target tissue to the frequencies of the motifs from the unselected library to test for randomness of distribution. We chose tripeptide motifs for the peptide insert analysis because three amino-acid residues seem to provide the minimal framework for structural formation and protein–protein interaction (Vendruscolo *et al.*, 2001). Comparisons of the motif frequencies in a given organ relative to those frequencies in the unselected library showed a nonrandom nature of the peptide distribution; such a bias is remarkable given that only a single round of *in vivo* screening was performed.

Of the tripeptide motifs recovered from tissues, some were preferentially found in a single site whereas others were recovered from multiple sites. This indicates that some of the recovered peptides home in a tissue-specific manner binding to differentially expressed endothelial cell markers in a given tissue while others bind to ubiquitous endothelial cell surface molecules. Further analysis of the original phage peptide inserts revealed four to six amino-acid residue motifs that were shared among multiple peptides isolated from a given organ. Each of these motifs were searched for similarities to known proteins in online databases, and found that some of the enriched peptide motifs appeared within known human proteins. Since our screening method isolates ligands for differentially expressed vascular receptors, our recovered peptide motifs are likely to mimic epitopes present in circulating ligands interacting with endothelial cell surface molecules. These circulating ligands may be either secreted proteins or surface receptors present on circulating cells interacting with the target tissue. We were able to identify a panel of candidate human proteins potentially mimicked by selected peptide motifs.

For example, one of the peptide motifs identified from the bone marrow is contained within bone morphogenetic protein-3B, which is a growth factor known to regulate bone development (Daluiski *et al.*, 2001). It is reasonable to expect that that the isolated peptide ligand mimics this protein. In addition to secreted ligands, motifs were also found in several extracellular or transmembrane proteins that may operate selectively in the target tissue, such as sortilin in fat (Lin *et al.*, 1997). We have also recovered motifs from multiple organs; one such peptide is a candidate mimic peptide of perlecan, a protein known to maintain vascular homeostasis (Nugent *et al.*, 2000). Similarly, we also identified IL-11 as a ligand mimicked by a peptide specifically enriched in the prostate tissue. IL-11 has been previously shown to signal through the IL-11 receptors within endothelium and prostate epithelium (Mahboubi *et al.*, 2000; Campbell *et al.*, 2001). This IL-11 mimicking peptide specifically bound to the endothelium and to the epithelium of normal prostate in phage overlay assay with human tissue sections; on the other hand, IL-11 mimetope failed to bind other organs, such as skin. In contrast, a phage isolated from the skin did not bind to prostate or other tissues; instead, this phage specifically identified blood vessels in the skin. Furthermore, the binding of the IL-11 mimetope peptide to IL-11Rα was verified *in vitro*. Validation of the of the ligand-receptor interaction has confirmed that our high-throughput identification of circulating peptide ligands does provide us with functional information in vascular biology in addition to organ homing ligands useful by themselves for vascular targeting. Additional support for the use of combinatorial screenings in patients for the development of anticancer targeted therapies comes from the studies by Zurita *et al.*, where IL11Rα was shown to be a potential target for intervention in human prostate cancer (Zurita *et al.*, 2004). Expression of IL-11Rα is increased in a stage-specific manner during disease progression in primary and metastatic prostate cancer and its associated blood vessels. Furthermore, a peptide guided by the IL-11 mimetope (CGRRAGGSC) peptide to the IL-11Rα linked to the well-established proapoptotic peptide $_{D}(KLAKLAK)_{2}$ (Ellerby *et al.*, 1999) was specifically targeted and internalized in to prostate cancer cells resulting in apoptosis (Zurita *et al.*, 2004).

Precedent exists to suggest that phage can be safely administered to patients, as bacteriophage were used in humans during the pre-antibiotic era (Barrow and Soothill, 1997). Ultimately, it may become possible to determine molecular profiles of blood vessels in specific conditions; infusing phage libraries systemically before resections of lung, prostate, breast and colorectal carcinomas, or even regionally before resection of limb sarcomas may yield useful vascular targets. Exploiting this experimental paradigm systematically with the developed analytical tools may permit the construction of a molecular map outlining vascular diversity in each human organ, tissue or disease. Translation of high-throughput *in vivo* phage-display technology may provide a contextual and functional link between genomics and proteomics. Based on the therapeutic promise of peptide- or peptidomimetic-targeting probes (Latham, 1999), clinical applications are likely to follow.

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